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Full Name of Author — Nom complet de l'auteur

IAN MACLEAN

Date of Birth — Date de naissance	Country of Birth — Lieu de naissance
22/03/58	CANADA

Permanent Address — Résidence fixe

#203 MICHENER PARK
EDMONTON, ALTA T6H 4M5

Title of Thesis — Titre de la thèse

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1983	Dr. Esmond J. Sanders

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CATIONIZED FERRITIN AND PHOSVITIN UPTAKE VIA COATED VESICLES IN EARLY CHICK EMBRYO CELLS

by

© Ian M. MacLean

A THESIS

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Ian MacLean

PERMANENT ADDRESS:

203 Michener Park
Edmonton, Alberta
T6H - 4M5

Dated Oct. 14 1983

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

"Cationized Ferritin and Phosvitin Uptake Via Coated Vesicles in Early Chick Embryo Cells"

submitted by Ian M. MacLean

in partial fulfillment of the requirements for the degree of Master of Science.

E. F. Sanders
.....
Supervisor

P. K. Chee
.....

A. E. Zglicki
.....

date 10/8/83

ABSTRACT

Stage 1 to Stage 5 chick embryos were examined using a transmission electron microscope. Coated pits (CP's) and coated vesicles (CV's) were observed in epiblast, mesoderm and endoderm cells. Cationized ferritin (CF) was employed as a marker for visualization of the distribution of negatively charged cell surface moieties. Binding of the marker was observed predominantly on the entire dorsal surface of the epiblast, with the exception of the deepest portion of the primitive groove in gastrulating embryos. Other cell surfaces, although accessible to the marker, bound relatively insignificant amounts of CF. However, when individual tissues were dissected from the embryo and cultured, all cell types bound CF to their surfaces in a in a manner similar to that seen on the dorsal surface of the epiblast *in situ*.

Embryos exposed to CF at 37°C prior to fixation bound the label in a clumped, or patchy distribution. This distribution was not apparently due to a post-binding rearrangement of ligand, as a similar binding pattern was observed on glutaraldehyde fixed embryos and embryos at low temperature (4°C).

Following binding to cells *in situ* and cells *in vitro* at 37°C, the CF was endocytosed via CP's and CV's and subsequently transferred to uncoated, irregularly-shaped membrane bound vesicles and multivesicular bodies. This process was

also observed to occur in the presence of the cytoskeletal inhibitors cytochalasin B, colchicine and vinblastine.

Native ferritin and horseradish peroxidase did not bind to any cell surface of whole embryos or tissue cultures, nor were they internalized by any of these cells, suggesting that bulk, fluid-phase endocytosis is very limited in these cells at this time in development.

The location of CP's on the dorsal surface of the epiblast in situ was, in some cases, apparently related to the presence of extracellular yolk granules bound to the cell surface. The yolk phosphoprotein phosvitin was covalently crosslinked to the ultrastructural marker ferritin, and both embryos and cultures were immersed in the conjugate. Binding of the conjugate was observed on exposed cell surfaces in both coated and uncoated regions at 4°C, and almost exclusively in coated membrane areas at 37°C, suggesting a degree of post-binding redistribution. Internalization via CV's ensued at 37°C, until after 10 minutes the conjugate was present in irregularly-shaped vesicular structures and multivesicular bodies. The binding and endocytosis of the conjugate was not inhibited by the presence of a thirty-fold excess of bovine serum albumin, but was inhibited by an equivalent excess of free phosvitin, suggesting that internalization of phosvitin occurs via receptor-mediated endocytosis.

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INTRODUCTION

During the course of gastrulation, the cells of the developing embryo undergo extensive changes in morphology, motility and molecular expression at the cell surface. Observing the spatial and temporal relationships of these dynamic characteristics provides a basis for hypotheses concerning their interdependence and respective functional roles in the development of the three-layered embryo. Such is the focus of the present study. By investigating characteristics of the early chick embryo, such as cell surface charge distribution and processes of nutritive uptake via endocytosis, an attempt has been made to further the understanding of some of the molecular processes underlying development.

The cell surfaces of the early chick embryo possess a net negative charge which is progressively reduced as development proceeds from Stage 1 through Stage 5 (Zalik et al, 1972). The results of the present study suggest that this is due to the conversion of an increasing proportion of the electronegative epiblast cells to mesoderm and endoderm, which appeared to bear practically no negative sites on their cell surfaces, as visualized in the transmission electron microscope by cationized ferritin (CF) binding. This change in cell surface charge occurred concurrently with respect to the developmental stage and spatial location of cellular passage

through the primitive streak.

Another interesting result of the present study was that the negatively charged cell surface moieties displayed a clumped distribution, which was apparently not due to a post-binding rearrangement of ligand, as has been shown to be true of certain ligands investigated in other systems (Schlessinger et al, 1978; Willingham et al, 1979). However, the functional significance of this natively clumped distribution of anionic cell surface charge remains obscure.

Previous investigations concerning the egg yolk of the chicken have partially characterized the yolk biochemically (Clark, 1977; Christmann, 1977) in conjunction with morphology (Bellairs and Backhouse, 1972). The receptor-mediated transport of nutrients such as phosvitin into the developing oocyte is now well documented (Cutting and Roth, 1973; Woods and Roth, 1979; Woods and Roth, 1981), and the uptake of yolk by the embryo via the newly developed extraembryonic circulation has been studied recently (Wobbs and MacMillan, 1981). Progress has been made concerning the suggested role of phosvitin in normal cellular events such as iron transport (Osaki et al, 1975; Hegenauer et al, 1979; Greengard et al, 1964) and oxidative phosphorylation (Grant and Taborsky, 1966). However, it was evident that the sequence of morphological events by which phosvitin is processed once it enters the cell, and indeed it if is in fact internalized by the early embryonic cells is at present

unknown. By tracing the pathway of internalization by ultrastructural labeling, it is hoped that this study can contribute to the understanding of this process.

The binding of phosvitin to the cell surfaces of the early chick embryo was rendered observable by conjugation of phosvitin to native ferritin. This conjugate was observed bound to exposed cell surfaces preferentially in areas overlying coated pits (CP's) at 37°C. This distinctive distribution was not evident following exposure to the conjugate at 4°C, indicating that at 37°C a post-binding rearrangement of ligand had taken place. Incubation of embryonic cells at 37°C in the presence of the conjugate resulted in internalization via coated vesicles (CV's) to intracellular vesicular compartments, including multivesicular bodies. Phosvitin, therefore, appeared to be internalized via the same pathway as the negative cell surface charge label CF. The endocytosis of CF was found, in the present study, to be independant of microfilament and microtubule function, as the process was not inhibited by colchicine, vinblastine or cytochalasin B.

LITERATURE REVIEW

Morphogenesis of the Early Chick Embryo

At the time of laying, the unincubated or Stage 1 (Hamburger and Hamilton, 1951) chick embryo is a disc of cells consisting of two layers, the epiblast and the hypoblast. The dorsal cell layer (epiblast) is separated from the underlying cell layer (hypoblast) by a cavity termed the blastocoele, and likewise the ventral surface of the hypoblast is seperated from the yolk by the subgerminal cavity.

The subgerminal cavity does not underlie the entire blastoderm, but only the central portion. The most ventral cells of the blastoderm periphery are in contact with the yolk. For this reason, the central area of the blastoderm appears more transparent than peripheral regions, and has been termed the "area pellucida", as opposed to the less transparent circumferential area which is referred to as the "area opaca".

Approximately five hours after the start of incubation (37°C), cells begin to accumulate in the posterior portion of the blastoderm. This thickening is called the "embryonic shield" which is soon transformed into a short, cone-shaped structure, known as the "initial streak", at the posterior edge of the area pellucida. This "initial streak" is the distinguishing feature of the Stage 2

chick embryo (six to seven hours incubated). At this point the streak exhibits a bipolarity of determination: the anterior half will give rise to embryonic mesoderm and endoblast, while the posterior half will contribute only to extraembryonic mesoderm (for review see Nicolet, 1971).

At this point the cells of the epiblast are rapidly moving toward the embryonic midline, as defined by the initial streak, and inward from the surface to further thicken and elongate the initial streak, until at twelve or thirteen hours of incubation it extends about halfway across the area pellucida. This is known as the "intermediate streak" of the Stage 3 embryo.

The streak continues to increase in length, mainly due to the movement of more anteriorly situated epiblast cells toward the midline. However, elongation is also thought to occur in a posterior direction (Vakaet, 1962), such that as the elongation progresses, the area pellucida loses its circular form and becomes pear-shaped, narrow at the posterior and broad at the anterior end. A groove becomes evident where the cells are inwardly migrating: the primitive groove. The primitive groove terminates anteriorly in what is referred to as the primitive pit. The primitive streak extends slightly anteriorly from the primitive pit in a clump of cells known as Hensen's node.

That the endoderm and mesoderm originate from epi-

blast cells which migrate through the primitive streak has been well established (Vakaet, 1970). More recently, scanning electron microscopy (SEM) studies on developing chick embryos have contributed greatly to the documentation of morphogenetic movements (Ebendal, 1976, Solursh and Revel, 1978, England and Wakely, 1977). From these studies the migration of cells through the primitive streak is apparently a result of a ventral extension of the flask-shaped cells in the deepest portion of the primitive streak, anchoring of these cells to subjacent cells by lamellipodia and filopodia, then directional cell shortening which pulls the cells down into the embryo's interior. A number of other changes have been shown to occur in cells passing through the primitive streak. As well as losing their epithelial morphology at this time, the electrophoretic mobility of the cells decreases at the primitive streak stage, as does their ability to bind Mg^{++} and Ca^{++} ions (Zalik et al, 1972, Harris and Zalik, 1974). As the absence of Ca^{++} and Mg^{++} ions is commonly used as a method of cell dissociation, these authors suggested that this was the means by which the cells became less tightly bound to one another during passage through the primitive streak. A decreased amount of cell surface fibronectin has also been suggested to contribute to this decrease in mutual affinity (Sanders, 1980). These cells subsequently contribute to the formation of both mesoderm and endoderm, the former of which

will first be considered.

The mesoderm migrates through the primitive streak, then forward and laterally into the cell-free space between the two cell layers, possibly due to a simple lack of contact-inhibition of movement in that direction, although the ever-present possibility of a diffusible chemical gradient has not been ruled out. Ebendal (1976) found no SEM evidence for the existence of structural fibrils of extracellular matrix which could direct the migration by contact guidance. More recently however, evidence derived from a combination of SEM, histochemical and immunofluorescence techniques has emerged in favor of a contact guidance system for primordial germ cells (Wakely and England, 1979).

The secretion of basal lamina on the ventral surface of the epiblast precedes the arrival of laterally migrating mesoderm cells. This evidence is in agreement with the conclusion of Trelstad et al (1976) that laterally migrating mesoderm used the ventral surface of the epiblast and the dorsal surface of the endoderm as substrates for locomotion, (for review see Bellairs 1982). The migrating mesoderm cells are associated with each other by lamellipodia and filopodia, and with endoblast and epiblast cells by filopodia. A large volume of cell-free space (between all mesoderm cells) is present throughout the mesodermal migration. As hyaluronate is the major component of the extracellular matrix synthesized at this time, and can have a hydrated volume 10^3 times larger than that of the

unhydrated state, it has been suggested that this glycosaminoglycan may be responsible for the appearance and maintenance of the cell-free spaces (Solursh, 1976). This claim has been augmented by a demonstration of decreased cell-free space in hyaluronidase-treated embryos (Fisher and Solursh, 1977). Moreover, it has recently been shown by autoradiography that hyaluronate synthesis begins within migrating cells during passage through the primitive streak (Vanroelen et al, 1980). The subsequent migration of each mesodermal cell has been suggested to occur via transitory attachments to endoderm and epiblast as well as other mesodermal cells. These attachments have been attributed to the binding of surface glycosyltransferases of one cell to oligosaccharides on the exposed surfaces of neighbouring cells (Shur, 1977), and attachment of cells to extracellular fibronectin (Sanders, 1980).

As mentioned previously, the endoblast also originates from epiblast cells which migrate through the primitive streak, although it is as yet unknown whether this occurs at the same time as migration of presumptive mesodermal cells through the primitive streak (for review see Bellairs, 1982). Recent SEM studies (Wakely and England, 1976, 1978) have confirmed earlier suggestions that a population of endoblast cells are already present, along with hypoblast cells, in the lower cell layer prior to gastrulation (Vakaet, 1962). This early endoblast

formation is a result of individual cells delaminating from the epiblast and positioning themselves among the cells of the hypoblast. As gastrulation proceeds more cells are added to the developing endoblast via the primitive streak. The constant addition of endoderm cells directly under the primitive streak has been suggested to push existing endoderm and hypoblast cells toward the periphery, such that endoderm there prior to gastrulation becomes a part of the area opaca (see also Rosenquist, 1972). The exact mechanism by which the endoblast formed during gastrulation invades the previously existing lower layer and inserts itself as a constantly expanding tissue patch is as yet unknown. However, in the Stage 5 (Hamburger and Hamilton, 1951) embryo the definitive endoblast is a well-established tissue layer underlying most of the area pellucida.

At the same time the mesoderm and endoderm are developing, the periphery of the epiblast is rapidly migrating away from the area pellucida, contributing to an overall expansion of the area opaca. These extra-embryonic epiblast migrating edge cells are the only epiblast cells which adhere to the fibrous, proteinaceous inner vitelline membrane (Bellairs et al, 1963) such that as expansion of the area opaca continues, the diameter of the ring of cell-substrate adhesion increases. The reason for the limited cell-substrate contact between the tissue and vitelline membrane has been purported to

be advantageous in the co-ordination of movement (Downie and Pegrum 1971). This idea is based on the assumption that an epithelial edge only one or two cells wide should be better able to make a co-ordinated directional movement than an epithelial edge many cells wide, simply because there are less cells to organize.

Labeling of Cell Surfaces with Cationized Ferritin

A polycationic derivative of ferritin, cationized ferritin (CF) is an electron-dense iron containing protein, used for labeling of negative charges on cell surfaces and was first synthesized in 1972 by Danon et al. This was accomplished by carbodiimide activation of horse spleen ferritin (average charge per molecule = -92) carboxyl groups followed by coupling to N, N dimethyl-1,3 propanediamine at pH 6.5. This imparted a charge of 2 to each carboxyl group, and average of 65 positive charges to each ferritin molecule.

Colloidal ferric oxide had been used as a label of cell surface negative charge previously (Gasic et al, 1968) but non-uniform particle size, low pH required for tissue treatment and the necessity for prefixation were drawbacks to this method. All of these difficulties were circumvented through the use of CF.

Danon et al (1972) established the usefulness of CF as an indicator of the negativity of cell surfaces through

an experiment involving rabbit red blood cells (RBC's). Young, old and neuraminidase-treated cells were compared with respect to electrophoretic mobility and CF binding capacity. The electrophoresis data and the CF binding data agreed that young RBC's were most negatively charged, followed by old RBC's and finally the neuraminidase-treated RBC's.

Since then, numerous studies have been conducted on various cell types to determine what kinds of cell surface anions are available for labeling by CF. Most often, these studies involve treatment of the cells with enzymes specific for a particular cell surface component, followed by exposure to CF, preparation for transmission electron microscopy, then comparison of CF binding distribution and density with controls. In one such study of the inner mitochondrial membrane of rat liver cells, (Hackenbrock and Miller, 1975) it was found that proteins, glycoproteins and glycolipids (especially sialoglycolipids) were all susceptible to CF labeling. Also it has been reported that about 50% of the anionic groups on the luminal front of the blood vessel endothelium of the guinea pig were sialic acid moieties and were removed by exposure to neuraminidase. Another study (Burry and Wood, 1979) attributed the capacity for CF binding on the cell surface of cultured rat cerebellum neuronal processes to the sialic acid moiety of glycoproteins and gangliosides, the carboxyl groups of proteins and the phosphate groups

of phospholipids. Nearly all of the CF binding capacity of sheep RBC's has been shown to be removed by neuraminidase (Marikovsky and Weinstein, 1981). The anionic sites located in coated pits on the luminal surface of mouse capillary endothelium are contributed only by proteoglycans and/or glycoproteins, whereas negative sites on the plasmalemma proper are of a more mixed chemical nature (Simionescu et al, 1981 b).

To summarize, it appears that a CF detectable negative cell surface charge may be due to the presence of a number of different types of anions. In most cells studied by these methods a large proportion of the net negative surface charge appears to be contributed by sialic acid, although it may vary tremendously between cell types. This variation has been exemplified using electrophoresis of chick embryo cells, the negative charge of which was shown not to be sensitive to neuraminidase (Zalik et al, 1972). Also, in a given cell type the negative surface charge in particular microdomains may be due to a proportionally different set of surface molecules than the remainder of the plasmalemma.

The pattern or distribution of CF to cell surface binding has been studied in a wide variety of cell types under many different experimental conditions. Exposure of glutaraldehyde-fixed cells to CF gives various results according to the type of cell studied. For example, CF binds in a random distribution to fixed baby hamster

kidney cells (Grinnell et al, 1975), the luminal surface of guinea pig blood vessel endothelium (Skutelsky and Danon, 1976) and rat liver mitochondrial membranes (Hackenbrock and Miller, 1975). CF binds preferentially to certain microdomains of the fixed luminal plasmalemma of mouse capillary endothelium (Simionescu et al, 1981 a.), isolated thyroid follicles (Denef and Ekholm, 1980) and to microvilli on fixed Erlich ascites tumour cells (Subjeck and Weiss, 1974). Also, CF binds in a rather uniform and continuous layer on the cell surface of fixed cultured embryonal cardiomyocytes (Lehner and Stevenson, 1981) and sheep erythrocytes (Marikovsky and Weinstein, 1981).

The CF binding distribution on the surfaces of unfixed cells at physiological temperatures also varies according to the cell type and the duration of exposure. However, generally there is a post-binding rearrangement of CF particles tending towards a greater degree of patching or clumping of the bound ligand with time (Borysenko and Woods, 1979, Subjeck and Weiss, 1974, Hackenbrock and Miller, 1975, Grinnell et al, 1975, Marikovsky and Weinstein, 1981, Lehner and Stevenson, 1981). One notable exception to this is the observation by Simionescu et al, (1981 a) that the preferential binding of CF to certain microdomains was observed in both fixed and unfixed cells. Skutelsky and Danon (1976) suggested the lateral migration or clumping observed in unfixed cells is a result of the cross-linking of the CF particles by negatively charged molecules.

Lehner and Stevenson (1981) have reported that CF arrested contractile activity in cultured cardiomyocytes according to the concentration of the CF dose. The arrest was suggested to be a result of a nearly complete occupation of cell surface Ca^{++} binding sites by CF molecules, as microfilament disruption could be suppressed by increased Ca^{++} concentration. Also, the arrest of contractile activity was correlated with an absence of gaps in the CF layer bound to the cell surface.

CF surface labeling techniques have been employed in attempts to elucidate the importance of the nature of cell surface charge. One example is the finding that B16 melanoma variants with differing lung colonizing potential had differing negative surface charge distributions (Raz et al, 1980). The fixed B16 melanoma cells which possessed a high lung colonizing potential showed large clusters of CF bound to the cell surfaces, while the low lung colonizing potential variants by and large showed a continuous layer where each CF molecule was separated from the adjacent molecule by about 100 Å. A second example is the report that the cell surface charge density decreased as slime mould cells approached agglutination (Lee, 1974). Differences in the distribution and mobility of cell surface anions on cultured secondary BALB/c embryonic fibroblasts and their SV-40 virus transformed counterparts have been found (Borysenko and Woods, 1979). Each cell type was subjected to a 10

second pulse label with CF, then fixed and observed. In the normal fibroblasts the label was clumped with large bare areas in between, the transformed cells exhibited a random distribution with very few small bare areas. If the cells were incubated for 20 minutes, following CF exposure, in CF-free saline the distribution of CF on the normal cells was not altered. Following the same treatment, CF was bound to transformed cells in very large clusters. Thus, it seems the anionic sites on the surfaces of the transformed cells have a more random inherent distribution and are more capable of long range mobility than their normal counterparts.

CF has also been used to investigate the process of membrane turnover in living mouse peritoneal macrophages (Skutelsky and Hardy, 1976). After these cells were exposed to CF <u>in vitro</u> most anionic sites disappear from the cell surface. These authors found that this disappearance was due to two processes, the concentration of CF-bound molecules to a part of the plasma membrane which is subsequently internalized, and detachment of CF-bound moieties from the cell surface. The capacity to bind CF was regenerated during incubation in CF-free tissue culture medium. This regeneration was shown to be regional at first, and not dispersed over the entire cell surface. This led the authors to suggest a mechanism for membrane turnover implicating insertion of whole patches of membrane by fusion of vesicles, rather than

dispersed insertion of membrane molecules.

In summary, CF binding is an established technique for examination of surface charge distribution. In the present work this label has been used in this way to determine surface charge density and distribution in various chick embryo tissues, in situ and in vitro.

Coated Pits, Coated Vesicles and Receptor-Mediated Endocytosis

Coated pits (CP's) and coated vesicles (CV's) are characterized by the proteinaceous latticework which surrounds them on the cytoplasmic surface of the plasma membrane. In a thin cross-section near the middle of the CV, the protein coat appears as tiny projections which are roughly perpendicular to the vesicle membrane. The relatively modern term "coated vesicles" dates back, in a definitive sense, to a study done by Rosenbluth and Wissig in 1964. The "coated pit" refers to the plasma membrane invagination which gives rise to the coated vesicle. In this paper they described the endocytosis of ferritin in the perikarya of toad neurons, which occurs via transport in CV's. More recently, CV's have been isolated from a variety of tissues (Pearse, 1975, 1976) by sucrose gradient centrifugation. In 1975, Pearse had subjected a partially purified aliquot of CV's derived from pig brain to sodium dodecyl sulfate polyacrylamide

gel electrophoresis, and found a major protein band of approximately 180,000 molecular weight. The protein was digested at the cytoplasmic surface of the vesicle by trypsin and pronase, and Pearse named it "clathrin". This molecule appears to have been highly conserved in structure between different tissues and different phylogenetic classes (Woods et al, 1978, Whyte and Ockleford, 1980).

It also has been shown that the protein lattice characteristic of CV's can be solubilized and thereby dissociated from the membrane by high concentrations of urea (Blitz et al, 1977), primary amines (Keen et al, 1979) or high pH (Woodward and Roth, 1978). This solubilized protein can be reassembled into basket-like structures which are morphologically similar to those seen on the original, intact CV's, that is a basket-like lattice of pentagons and hexagons. Electron micrographs of the solubilized protein have been obtained and from these the basic assembly unit of the cage appears to be a pinwheel-like structure with three-fold symmetry (Ungewickell and Branton, 1981). This assembly unit consists of three heavy protein chains of 180,000 molecular weight and three light chains (at least two species of which have been identified, with molecular weights of 33,000 and 36,000). Each heavy chain is bound to one light chain, and the complete unit is a trimer of

these pairs, thus the three-fold symmetry. These pinwheel-like trimers appear to be the building blocks of the protein coats of CV's (Kirchhausen and Harrison, 1981).

Pearse (1975, 1976) also performed an analysis of the lipid composition of the CV membrane. In these studies it was discovered that the cholesterol to phospholipid molar ratio of CV membrane was significantly lower than that found in plasma membranes, and more closely approximated the ratio commonly found in internal membranes. The same is true of lipids isolated from chicken oocyte CV membranes (Whyte and Ocklefore, 1980). Therefore, it seems that coated endocytic vesicles may selectively internalize lipids from the plasma membrane, such that cholesterol-rich areas are excluded, leaving the cholesterol to phospholipid molar ratio of the target organelle/s undisturbed.

The distribution of CP's on the surfaces of cells ranges from a seemingly random distribution in mouse fibroblasts (Bretscher et al, 1980) to a distinct regional confinement (Pfeiffer et al, 1980) in mouse macrophages.

The most extensive research pertaining to CV function has been concentrated in the area of their involvement in the receptor-mediated endocytosis of proteins, although they are also known to participate in secretory processes (Kartenbeck, 1980), membrane recycling (Steinman et al 1976, Schneider et al 1979) and intracellular transport (Friend and Farquhar, 1967). Such molecules as immunoglobulin G

and vitellogenin in developing chick oocytes (Roth et al, 1976), epidermal growth factor, or EGF (Haigler et al, 1979), low density lipoprotein, or LDL (Anderson et al, 1977) and α_2-macroglobulin (Willingham et al, 1979) in human fibroblasts, have been observed to enter the cells via CP's and CV's. In some of these studies, conjugation of the protein being studied to an ultrastructural marker such as ferritin or horseradish peroxidase (HRP) allowed direct electron microscopic visualization of different stages in the endocytotic process.

The first stage of receptor-mediated endocytosis involves the binding of an external ligand to a cell-surface receptor. This binding is a specific interaction and has been studied from the point of view of receptor distribution before and after binding of the ligand.

The receptor-mediated endocytosis of LDL by human fibroblasts is one of the most thoroughly investigated of these systems (Anderson et al, 1977). The unbound LDL receptors on the fibroblast cell surfaces were found to be widely but unevenly distributed in the plane of the plasma membrane. They observed that 60 to 70% of the ferritin labeled LDL that bound to the cells, at 4°C (where lateral movement of bound ligand is retarded) and on fixed cells, was localized in CP's which make up no more than 2% of the total surface area. Wall and Hubbard (1981) obtained similar results through observation of asialoglycoprotein receptors on the cell surfaces of rat hepatocytes. When cells exposed to labeled ligand at 4°C

were subsequently warmed to 37 C, within 10 minutes nearly all the bound ligand was present in CV's, which presumably had formed from the "pinching off" of CP's. However, evidence has since been presented indicating that the coated pits of fibroblasts do not pinch off at all, but simply concentrate the bound ligand (α_2-macroglobulin) and transfer it to an uncoated vesicle. This uncoated vesicle, called a "receptosome", forms immediately adjacent to the CP and is purported to be responsible for the internalization of the ligand previously concentrated in the CP (Willingham and Pastan, 1980). In other words, what may in some thin sections appear to be a fully enclosed CV, is observed in serial sections to be continuous with the plasma membrane via a long, narrow "neck", and therefore still open to the external milieu.

Anderson et al (1977) have additionally identified a mutant with an impaired ability to localize LDL receptors in CP's. The LDL receptor is believed to be a transmembrane protein and this mutant is thought to synthesize receptors defective in the region of the molecule which normally associates with clathrin on the cytoplasmic membrane surface. This defect manifests itself in the pathological condition known as familial hypercholesterolemia, as does the inherited lack of the entire LDL receptor. In this study they also obtained evidence that in normal human fibroblasts the LDL receptors were recycled following internalization in CV's, as internalization and replenishing

of surface receptors continued for at least six hours when protein synthesis was effectively inhibited with cycloheximide. Other evidence for the occurrence of a cell surface component recycling phenomenon has been obtained using CF and HRP as ultrastructural markers (Thyberg et al, 1981). Following a pulse label with HRP and a subsequent chase with normal medium, HRP was observed to accumulate in lysosomes via adventitious incorporation into endocytic vesicles. At this point no HRP was present on the cell surfaces. Then the cells were exposed to CF which bound to the plasma membranes, and was internalized in endocytic vesicles and transferred to lysosomes. Subsequently, CF-HRP complexes were observed in Golgi cisternae, cytoplasmic vesicles and bound to the cell surface.

Although these ultrastructural markers have proven to be valuable in many studies, their use as labels conjugated to ligands whose endocytosis it is desired to follow may be responsible for the specificity of binding, and therefore characteristics of internalization, in some cases (Wild, 1980). Therefore, the appropriate experiments must be done to eliminate these possibilities when a study of this kind is undertaken.

As previously mentioned, the LDL receptors of human fibroblasts are preferentially located in CP's prior to ligand binding, however this premise can not be expanded to encompass receptor-mediated endocytosis in general. For example, ferritin-conjugated α_2-macroglobulin binds

to fixed fibroblasts in culture with an entirely diffuse distribution (Willingham et al, 1979). When this conjugate was exposed to unfixed cells for 5 minutes at 4°C, a diffuse pattern of labeling was again observed, only some clustering of bound ligand into CP's was evident, indicating that mobility of molecules in the plane of this membrane is not completely retarded at this temperature. Furthermore, when the conjugate was exposed to cells at 37°C for 1 minute prior to fixation, the receptor-ligand complexes were rapidly redistributed into coated membrane areas.

Further variations of the mechanism of receptor-mediated endocytosis in fibroblasts have been elucidated in studies involving fluorescent derivatives of epidermal growth factor (EGF) and insulin (Schlessinger et al, 1978). In this study it was found that both EGF and insulin receptors are natively diffusely distributed in the plane of the plasma membrane. However, this is not to say that they are randomly distributed, as about 25% of bound ferritin-EGF conjugate was located in groups of 5 molecules or more (Haigler et al, 1979), and unoccupied insulin receptors appear in groups of two to six molecules (Anderson et al, 1977). There is no post-binding rearrangement of these receptors at 4°C, however, the hormone-receptor complexes became aggregated into patches, immobilized and internalized within a few minutes at 37°C. These observations were attributed to either hormone to

hormone cross-linking or increased affinity of receptors for one another, caused by a conformational change in the receptor following binding of ligand (Schlessinger et al, 1978).

It has been found that CP's and CV's not only act as sites of concentration and internalization of specific ligands, but additionally may preferentially exclude certain membrane proteins from their confines (Bretscher et al, 1980). This evidence agrees very nicely with the idea that CV's mediate the transfer of specific molecules between intracellular organelles, without transporting membrane proteins specific to a particular organelle.

Up to this point no mention has been made, in this review, of the eventual fate of the endocytotic CV's and their contents. This area has been extensively studied and overall, has been found to be extremely variable between cell types. Although a complete compilation of the destinations of endocytic CV's in all cell types studied and the suggested reasons behind them is beyond the scope of the present work, a rough idea of the extent of this diversity will be given here. For example, endocytic CV's have been shown to travel through the cytoplasm and fuse with other CV's, MVB's, primary or secondary lysosomes, Golgi elements or the plasma membrane depending on the cell type and the nature of the ultrastructural marker used to trace their fate (for review see Nevorotin, 1980). In some cases, several of these pathways may be traced by the same label, in the same

type of cell (Van Deurs et al, 1981). The clathrin lattice may or may not be completely lost prior to the fusion initiation. In still other instances, as previously mentioned, it appears that the CP's on the cell surface do not morphologically separate from the plasma membrane, but transfer their contents to a developing receptosome which subsequently pinches off leaving the coated pit still attached to the cell surface (Willingham et al, 1981). The receptosome then moves through the cytoplasm to a position near the Golgi apparatus, and then delivers its contents into newly formed lysosomes (Willingham and Pastan, 1980).

In summary, CP's and CV's appear to mediate the transport of certain molecules to specific sites in the cells of a wide variety of tissues. The presence of the clathrin coat has been suggested to mediate this specificity, preventing transfer of the vesicular contents to inappropriate sites and/or facilitating recognition and subsequent fusion with the target membrane (see Ockleford and Munn, 1980).

Avian Egg Yolk: Deposition and Utilization

In many instances, yolk deposition involves selective protein transport from the maternal circulation to the interior of the developing oocyte (Roth et al, 1976, Wallace and Dumont, 1968, Jared et al, 1973). This

selective protein transport is mediated by CP's and CV's and has been demonstrated to be the pathway of internalization into the oocyte for the maternal serum protein vitellogenin, as well as immunoglobulins, very low density lipoprotein and low density lipoprotein in the chicken oocyte (Cutting and Roth, 1973, Roth et al, 1980). This sequestration of proteins is known as vitellogenesis, and from chicken oocyte membranes a receptor, specific for vitellogenin, has been solubilized and characterized (Woods and Roth, 1979). The chicken vitellogenin molecule is thought to consist of two phosvitin subunits and one lipovitellin subunit (Christmann et al, 1977). It has recently been demonstrated, through competitive binding studies, that the binding of vitellogenin to its receptor on the oocyte membrane, is mediated by its phosvitin moiety (Woods and Roth, 1981). Following endocytosis of the ligand-receptor complex, the clathrin coat is lost from the cytoplasmic surface of the vesicles which then undergo fusion with larger protein-containing vesicles. The vitellogenin is broken down in the oocyte to yield the lipovitellin and phosvitin, which remain associated with each other in the discrete particles which constitute the "granule fraction" of avian egg yolk (Bellairs et al, 1972). The role of phosvitin in chick development is believed to be that of a rich source of iron and phosphorous for the developing embryo (Clark, 1977, Hegenaur et al, 1979).

The uptake of yolk proteins by the developing chick embryo has long been an active field of study (for review see Schechtman, 1956). On the basis of these studies it has been generally accepted that the cells of the area opaca actively phagocytose extracellular yolk, however there is little evidence to indicate that this also occurs within the area pellucida (Bellairs, 1958, Bellairs and New, 1962). What has been established through tracing of ferritin and HRP endocytosis is that the endoderm of the yolk sac may take up yolk through sequestration in CP's and CV's which retained their clathrin coats until fusion with apical vacuoles (Mobbs and McMillan, 1981). The specificity of the uptake of yolk constituents in this manner has not been established, although these authors note that CP's in the yolk sac endoderm are associated consistently with dense extracellular yolk granules and, "...although there may be no specific selection of an individual protein, endodermal cells may endocytose a certain yolk fraction composed of more than one protein".

The apical vacuoles have been suggested to contain lysosomal enzymes which play a role in the digestion of endocytosed extracellular materials (Lambson, 1970, Mobbs and McMillan, 1981). Following incorporation into apical vacuoles, the tracers HRP and ferritin were observed in pleomorphic yolk drops deeper within the cytoplasm and subsequently in the vitelline circulation. The exact mechanism of this transfer could not be determined in

this study but it was proposed that deposition of molecules into the intercellular space below the areas of junctional contact between cells could account for this, as ferritin particles were observed in these spaces.

The intracellular breakdown of yolk in the early chick blastoderm has been examined by transmission electron microscopy (Bellairs, 1958). In this study it was concluded that the area pellucida of the early chick embryo (unincubated through 10 pairs of somites) contains 3 main types of yolk drop. These 3 types were termed "complex yolk drop", "type A yolk drop" and "type B yolk drop". Complex yolk drops were purported to be early stages in yolk conversion and contained both type A and type B yolk drops. In time, the membranes around the complex yolk drops break down, releasing type A and type B yolk drops into the cytoplasm. The type A yolk drops then appear to be converted into MVB's, the limiting membranes of which eventually break down releasing the circular, or possibly tubular bodies or microparticles into the cytoplasm where they may contribute to endoplasmic reticulum or cytoplasmic microparticles. The type B yolk drops, believed to be primarily lipid gradually become smaller and fewer in number but their eventual fate was not ascertainable.

It is therefore apparent that while some aspects of yolk deposition and utilization have been elucidated, much remains to be discovered in this field. Part of

the present study is therefore directed toward a further understanding of yolk utilization via endocytosis in the area pellucida of the early chick embryo.

The Cytoskeleton, Receptor Distribution and Endocytosis

Microfilaments (MF's) and microtubules (MT's) have been implicated in numerous cellular processes, including the motility of cells as a whole and of their constituent organelles and particles. Evidence for the involvement of MF's and, to a lesser extent MT's in the distribution of cell surface components has largely been obtained through studies of "capping" phenomena (for review see Koch, 1980). Capping refers to the "ligand induced polar redistribution of cell surface receptors" (Taylor et al, 1971). A primary requirement of capping is the use of a multivalent ligand capable of cross-linking cell surface receptors. This cross-linking appears as patches of clustered receptor-ligand complexes, and following this, these patches are translocated to one pole of the cell in a process requiring metabolic energy.

One example of the capping phenomenon is the response of fibroblasts to the lectin concanavalin A. The involvement of microfilaments in this process has been supported by electron microscopic demonstrations of close morphological proximity between concanavalin A caps and large amounts of actin (the MF protein) in the cortical cytoplasm

directly underlying the cap (see Condeelis, 1980). Also, the relationships between MF's, MT's and cell surface receptor capping have been extensively investigated using drugs which are known to disrupt cytoskeletal elements. Colchicine, which disrupts MT's and cytochalasin B, which disrupts MF's are two such widely used agents.

The effects of colchicine on capping are extremely variable between experimental systems, as it may have no effect, an inhibitory effect or an enhancing effect, however the latter case appears to be the most prevalent. Cytochalasin B, however, always exerts some degree of an inhibitory effect on capping.

At present, the most widely accepted model to account for these observations is known as the "anchorage model", which envisages receptors to be "held in place" by MF's to create an energy barrier to receptor clustering known as the nucleation threshold (for review see Rees et al, 1980). When the MF's are disrupted this threshold is theoretically lowered, allowing co-operative binding of ligand and resultant patch formation; however, cap formation is inhibited. The role of MT's in cap formation is generally not well defined due to the variability of the effects of colchicine.

Other lines of evidence also indicate an association between cytoskeletal elements and cell surface components. For example, both cell surface H antigen and cell surface immunoglobulin have been shown to co-purify with MF's in

some cells (see Condeelis, 1980). Also, proteolysis confined to the external cell surface results in disaggregation of intracellular MF bundles (see Rees et al, 1980). In summary, it has been well established that cellular MF's and MT's are capable of influencing cell surface receptor distribution, however, the nature of the interactions on a molecular level are not well understood.

The effects of cytoskeletal disruption on endocytosis are extremely variable depending on the type of endocytosis and the particular example studied (for review see Silverstein et al, 1977). It appears that the only consistent generalization to be drawn from the literature is that cytoskeletal disruption does not enhance endocytosis in any system.

To elaborate on the variability of the effects of both colchicine and cytochalasin B on endocytosis several examples will be considered. Colchicine inhibits fluid-phase endocytosis of HRP and adsorptive endocytosis of cationized HRP in mouse peritoneal macrophages (Thyberg and Stenseth, 1981) and the absorptive uptake of colloidal gold by rat peritoneal macrophages. However, the fluid-phase uptake of polyvinylpyrrolidone in rat peritoneal macrophages was inhibited only at concentrations where the specificity of the inhibitor was in doubt, (Pratten and Lloyd, 1979) as at high concentrations colchicine inhibits transmembrane nucleotide transport

and has a hypocalcemic action (Allison and Davies, 1974). Cytochalasin B has no effect on the fluid-phase endocytosis of HRP or the adsorptive endocytosis of cationized HRP by mouse peritoneal macrophages (Thyberg and Stenseth, 1981) or on the adsorptive pinocytosis of ferritin or colloidal gold by mouse macrophages (see Silverstein et al, 1977). However, cytochalasin B inhibits the adsorptive pinocytosis of lysosomal hydrolases and sulfated glycosaminoglycans by skin fibroblasts (von Figura et al, 1978) and the adsorptive uptake of colloidal gold and fluid-phase endocytosis of polyvinylpyrrolidone by rat peritoneal macrophages (Pratten and Lloyd, 1979).

With particular reference to pinocytosis involving CP's and CV's, it has been found that cytochalasin B interferes with this process. For example, investigators have found that in human lymphoblastoid cells, cytochalasin B inhibits the conversion of CP's to CV's (Salisbury et al, 1980). This would infer a functional role of microfilaments in coated vesicle formation. However, cytochalasin B has been found to have no effect on the uptake via CP's and CV's of I^{125}-labeled asialoglycoprotein by rat hepatocytes (Kolset et al, 1979).

In summary, the effects of cytoskeletal inhibitors on various endocytic systems have been shown to be variable between cell types and different endocytic markers in a given cell type. The present study will therefore attempt to ascertain the degree of involvement of the cytoskeletal elements in the endocytic processes

of the early chick embryo.

MATERIALS AND METHODS

1) Preparation of Embryos

The fertilized eggs of White Leghorn chickens were purchased from the Poultry Farm, University of Alberta. The embryos were dissected out of the eggs and freed of adhering yolk and vitelline membrane in 0.1M phosphate buffered saline (PBS) pH 7.4 at room temperature. In some embryos the endoblast was removed from one half of the embryo, to allow access of chemicals to the mesoderm and ventral surface of the epiblast.

The 0.1M PBS was prepared by mixing 7.2g NaCl, 1.48g Na_2HPO_4 and 0.43g KH_2PO_4 in one liter of distilled water. The pH was adjusted to 7.4 with NaOH and the buffer was stored at room temperature.

Following dissection, the embryos were thoroughly rinsed in PBS and were deployed in various ways according to the requirements of each experiment.

2) Transmission Electron Microscopy, (TEM)

Following exposure to a particular set of experimental conditions, embryos and cultures were rinsed three times for five minutes each with PBS, then fixed in 2.5% glutaraldehyde (Ladd Research Industries, Burlington, Vt.) in 0.1M PBS for 4 hours at the temperature required by the experiment. Then they were

rinsed again, as above, and post-fixed in 1% OsO_4 in 0.1M PBS pH 7.4 for 1 hour at room temperature. Rinsing was repeated and followed by dehydration in a graded series of ethanol (10 minutes in each of 50%, 70%, 85%, 95% and twice in absolute ethanol). The specimens were then immersed in propylene oxide for 20 minutes before being placed in a 1:1 mixture of propylene oxide and Araldite embedding resin (Luft, 1961). The specimens remained in this mixture overnight at room temperature, then the mixture was drained off and replaced by pure Araldite for 6 to 8 hours. Embryos were then placed in a rubber embedding mould, covered with pure Araldite and incubated at 60°C for 48 hours. In the case of cell cultures, these were covered with pure Araldite in the culture dish and incubated at 60°C for 48 hours. The plastic dish was then removed and the areas of the block containing cells were cut out. These blocks were then inverted such that the side which formerly contacted the plastic dish was dorsal, placed in rubber moulds and covered with pure Araldite. After a further polymerization at 60°C for 48 hours the blocks were trimmed with a razor blade.

Thin sections showing a silver interference pattern (approximately 600 A) were cut using glass knives on either a Sorvall Porter-Blum Mt-2 ultramicrotome or a Reichert OmU2 ultramicrotome. Sections were picked up on formvar coated 200 mesh copper grids.

Unless the nature of the experiment required unstained sections, sections were stained with 5% uranyl acetate in absolute methanol for 10 minutes, rinsed in 4 changes of absolute methanol and counter-stained with Reynolds lead citrate (Reynolds, 1963) for 60 seconds. Grids were examined with a Phillips EM 300 electron microscope at 60 KV.

For light microscopy, the same procedure as for TEM was followed up to and including block trimming. Following this, thick sections of approximately 1 thickness were cut on a Reichert OmU2 ultramicrotome using glass knives. Thick sections were placed on glass microscope slides, dried, then stained with Richardson's stain for 60 minutes on a hotplate preheated to 115°C. Stained sections were examined and photographed using a Leitz Orthoplan light microscope mounted with a Leitz Orthomat camera.

3) Scanning Electron Microscopy (SEM)

Embryos were prepared for SEM examination in the same manner as described for TEM preparation up to and including ethanol dehydration. They were then transferred to vials containing 100% acetone, which was changed twice at 10 minute intervals, stoppered and stored at 4°C overnight. Critical point drying from CO_2 was carried out, followed by coating the samples with gold and examination with a Philips 505 microscope.

4) Tissue Culture

All tissue samples were taken from Stage 5 (Hamburger, and Hamilton, 1951) chick embryos using sterile technique. Approximately 0.25 mm squares of either endoblast, mesoblast or epiblast were dissected out of the embryo using Tungsten needles in 0.1M PBS pH 7.4. The separate tissue samples were explanted via silicon-coated narrow mouth Pasteur pipette into Earle's 199 medium containing 10% fetal calf serum (FCS, GIBCO, Grand Island, New York) and 5% Penicillin (500 units) Streptomycin (500 mcg/ml) GIBCO in drops in Permanox plastic dishes (LUX Scientific Corporation, Newbury Park, Calif.). Sterile PBS drops were placed around the inside periphery of the dish to suppress evaporation of the culture medium. The dishes containing the explants were then placed into a 4% CO_2 environment and incubated at 37°C for 24 hours. All explants were then examined under a Leitz inverted phase-contrast microscope and those which had not attached to and spread on the substratum were discarded.

5 Preparation of Phosvitin-Ferritin Conjugate

The method for synthesizing this electron-dense marker (Avrameas, 1969) consisted of mixing 12.5 mg of phosvitin (PV), having a molecular weight of about 34,000, and 162.5 mg of ferritin (mol. wt. 450,000) in 5 ml of 0.1M PBS (phosvitin and ferritin from Sigma Chemical Co., St. Louis, Mo.), .25 ml of 1% glutaral-

dehyde is added dropwise and further mixed for 1 hour at room temperature.

After the conjugation reaction the solution was transferred to a dialysis bag (pre-boiled in distilled H_2O for 2 hours) and dialyzed against a large volume of buffer for 2 hours with constant mixing. Then the buffer was drained and replaced with fresh buffer and dialyzed overnight, to remove any remaining free glutaraldehyde drom the dialysate.

The solution was then applied to a Sephadex G-75 gel exclusion cloumn equilibrated with 0.1M PBS pH 7.4. The conjugate-containing fraction was collected immediately after the void volume. The fraction corresponding to free PV peak was collected and a protein concentration determination was conducted, according to the method of Bradford (1976) using the dye Coomassie Brilliant Blue G-250 purchased from Bio-Rad Laboratories, Richmond Calif. USA. The concentration in the PV peak was found to be less than 5 g/ml. The total protein-containing volume after the conjugate peak was 180 ml, as determined by monitoring the absorbance at 280 mm with an Isco type 6 optical unit, model 328 fraction collector and VA-5 absorbance monitor. This gave the maximum amount of PV outside the conjugate peak to be less than 0.9 mg. Subtraction of this value from the original amount of PV used to synthesize the conjugate gives a minimum of 11.4 mg of PV present in the conjugate fraction of 40 ml. Therefore, the concentration of PV-ferritin complex was

was not less than 0.25 mg/ml.

6) Experimental Procedures

Cationized Ferritin (CF) Experiments

Sterile cationized ferritin (11mg/ml) in 0.15M NaCl was purchased from Miles Laboratories Inc., Elkhart, Ind. In one experiment, CF was diluted to 0.12 mg/ml with PBS, in all others 0.25 mg/ml was the concentration employed. Embryos were transferred to vials containing about 2 ml of CF solution via wide-mouth pipette.

Immediately prior to, and following CF exposure all embryos (Stages 1, 3 and 5 according to Hamburger and Hamilton, 1951) and cultures were rinsed thoroughly with buffer. Exposure conditions varied with regard to temperature (4°C or 37°C) and time of exposure (1 min. to 60 min.) according to the aim of the particular experiment. In some experiments, when fixation of embryos in 2.5% glutaraldehyde in PBS pH 7.4 preceded CF exposure, embryos were rinsed in 5% glycine in PBS to neutralize residual free aldehydes prior to CF exposure.

To be sure of a homogenous solution of free CF particles, rather than aggregates, a drop of the CF solution was placed on a coated grid and allowed to dry. Subsequent electron microscopic examination revealed a scarcity of aggregates and a vast majority of free CF molecules.

Ferritin Experiments

Sterile Type 1 horse spleen ferritin (100 mg/ml) in 0.15M NaCl was purchased from Sigma Chemical Co., St. Louis, Mo. The ferritin was diluted to 0.25 mg/ml or 5 mg/ml with 0.1M PBS, pH 7.4. Stage 5 embryos and cultures were exposed to the ferritin solution at one of the above concentrations for times ranging from 5 to 30 minutes at 37°C or 4°C and then rinsed with buffer and processed for TEM.

Horseradish Peroxidase (HRP) Experiments

Type VI Horseradish Peroxidase was purchased from Sigma Chemical Co., and was dissolved in 0.1M PBS pH 7.4 at a concentration of 2 mg/ml. Stage 5 embryos were exposed to the HRP solution for 10 min. or 30 min. at 37°C or 4°C and subsequently rinsed in buffer, and fixed with 2.5% glutaraldehyde at 4°C overnight. They were then rinsed thoroughly with PBS and exposed to 3, 3'-diaminobenzidine (DAB, J.T. Baker Chemical Co., Phillipsburg, N.J.) at a concentration of 0.5 mg/ml in PBS for 1 hour at room temperature. Following another thorough rinse in PBS, embryos were further processed for EM in the usual manner.

Phosvitin and Phosvitin-Ferritin Conjugate Experiments

To assess any morphologically detectable response to the presence of extracellular PV, Stage 5 embryos and

tissue cultures from each of the three germ layers, were exposed to either 0.05 mg/ml PV or 0.5 mg/ml PV in 0.1M PBS, pH7.4, for 15 min. at 37°C. Controls were incubated in PBS only. Following this treatment the cells were rinsed thoroughly in PBS and processed for EM.

To investigate the possibility of PV binding to the plasma membranes and then being endocytosed, a PV-ferritin conjugate was employed. Both whole embryos and tissue cultures were exposed to the conjugate solution for 10 min. at 37°C or for 10 min. at 4°C. Controls underwent the same incubations in pure PBS. Following these treatments all specimens were rinsed thoroughly with PBS and processed for EM.

To test the specificity of the conjugate-membrane interaction, not less than a thirty-fold excess of free PV was dissolved in the conjugate solution and the experiment was repeated as above. In controls, not less than a thirty-fold excess of BSA was dissolved in the conjugate solution in substitution for free PV.

Cytoskeletal Inhibitor Experiments

The significance of microtubules and microfilaments in the binding and endocytosis of CF was investigated using cytochalasin B (CB), colchicine and vinblastine (all from Sigma Chemical Co.). CB was dissolved in 0.1% v/v dimethylsulphoxide in PBS, pH 7.4, to a concentration

of $2x10^{-5}$ M. Stage 5 embryos were immersed in vials containing about 2 ml of CB solution for 2 hours at room temperature. The embryos were then warmed to 37°C and CF was added to the CB solution to a concentration of 0.25 mg/ml. Incubations in this mixture was of 5, 15 or 30 min. duration, followed by processing for EM. Control embryos received identical treatment in the absence of CB.

Colchicine and vinblastine experiments were performed in the same manner as the CB experiments, again at a concentration of $2x10^{-5}$ M in 0.1M PBS, pH 7.4, the only difference being the absence of dimethylsulphoxide.

RESULTS

1) Morphology of the Stage 5 Chick Embryo

In low magnification Scanning electron micrographs of the Stage 5 chick embryo the primitive streak is observed to extend about three-quarters of the anterioposterior length of the area pellucida (Fig. 1), terminating anteriorly in the primitive pit. The entire area pellucida was elongated in the anterioposterior direction and topographically distinguishable from the area opaca.

In fractured specimens, the irregularly shaped mesoderm cells were observed sandwiched between the columnar epiblast and flat endoblast (Fig. 2).

Observation of the dorsal surface of the epiblast at higher magnification (600X) revealed the presence of a great many spherical structures of extremely variable size (Fig. 3). These spheres were in greatest abundance within the primitive groove, although the entire dorsal surface of the epiblast appeared littered with them. These spheres were certainly yolk granules since upon examination of the yolky ventral surface of the area opaca, yolk spheres of similar sizes and appearance were observed (Fig. 4).

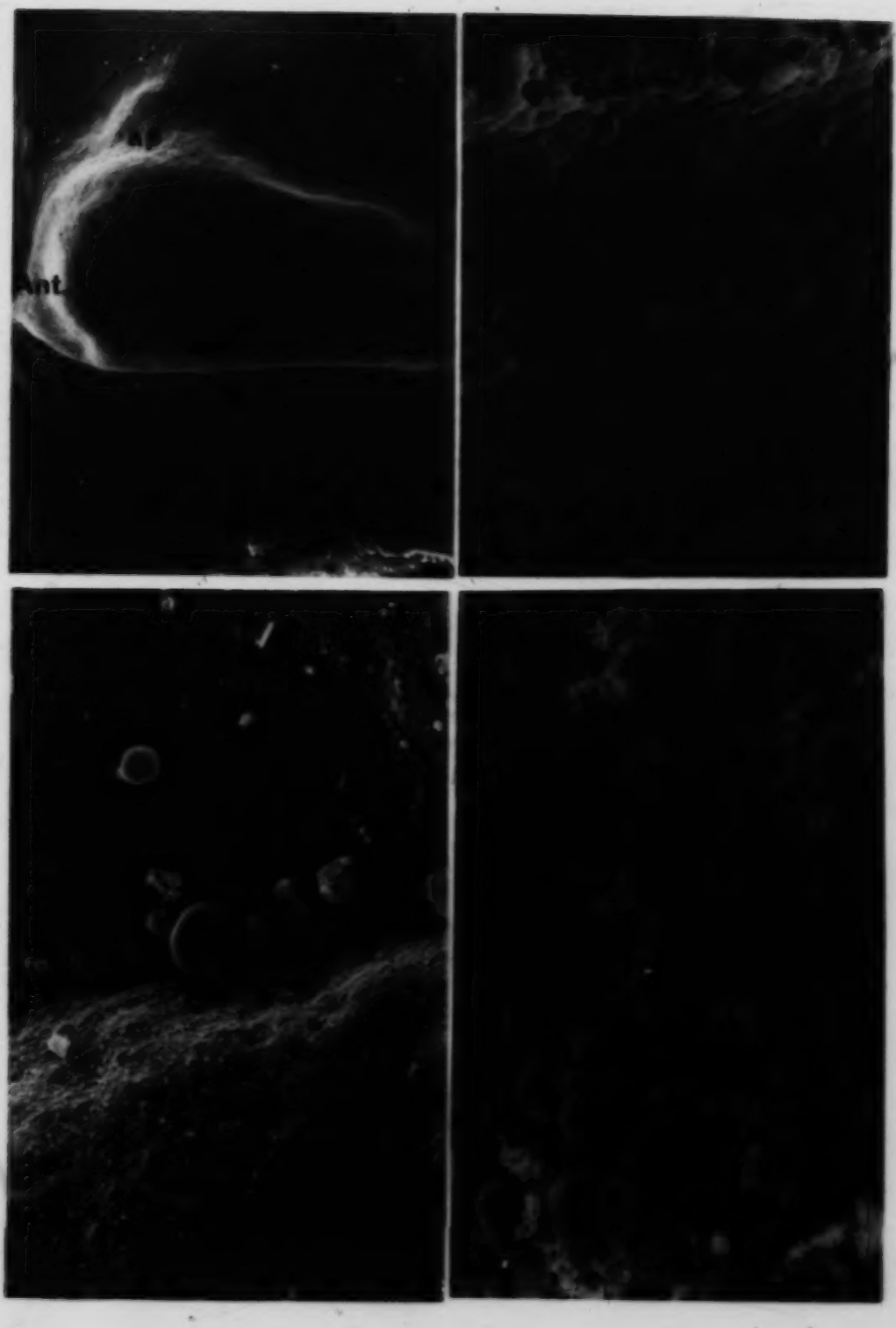
For light microscopy, thick transverse sections were taken approximately from the anterioposterior mid-point of the primitive streak. The light micrographs

Fig. 1 Scanning electron micrograph (SEM) of whole Stage 5 chick embryo dorsal view illustrating the primitive streak (PS), area pellucida (ap), area opaca (ao) and primitive pit (pp). Anterior (Ant.) end of embryo is specified X 29.

Fig. 2 TEM near the middle of fractured Stage 5 chick embryo illustrating the spatial relationship between the three germ layers: epiblast (EPI), mesoderm (MESO) and endoderm (ENDO) X 600.

Fig. 3 SEM of primitive streak of Stage 5 chick embryo showing many yolk spheres of variable size, bound to the dorsal surface of the epiblast in the primitive streak (PS) area. X 600.

Fig. 4 SEM of yolk spheres adhering to the ventral surface of the area opaca, X 600.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(Fig. 5) taken of these sections show the cells of the migrating mesoderm and large cell-free spaces between the columnar epiblast and flat endoblast cell layers. Cells about to undergo migration through the primitive streak are evident as "bottle-shaped" cells, that is, the apical side is narrow and the basal side wider.

2) Occurrence of Coated Pits and Coated Vesicles

In the chick embryo, coated pits (CP's) and coated vesicles (CV's) are identifiable in the transmission electron microscope by the presence of the clathrin coat which appears as an array of bristle-like projections on the cytoplasmic surface of the pit or vesicle membrane (Figs. 6 and 7). These CP's and CV's were observed in cells from all areas and tissues of the area pellucida in all stages of embryonic development examined in this study (Stages 1, 3 and 5). CP's were observed on the dorsal and ventral surface of the endoblast, on mesoderm cell surfaces and on the dorsal and lateral surfaces of the epiblast cells (Fig. 7), including those in the primitive streak area. Frequently, CP's were observed in immediately adjacent locations on the lateral surfaces of two neighboring epiblast cells (Fig. 8). Also, in many instances where a quantity of the granule fraction of the yolk was seen closely apposed to the plasma membrane of the epiblast, a CP or CV was observed in the portion of the membrane most closely

associated with it

Fig. 5 Light micrograph of thick, transverse section (1) taken from near the antero-posterior mid-point of the primitive streak illustrating the epiblast (epi), mesoderm (meso), endoderm (endo) "bottle-shaped" cells (marked by arrowhead) and cell-free spaces (some examples indicated by arrows). X 50.

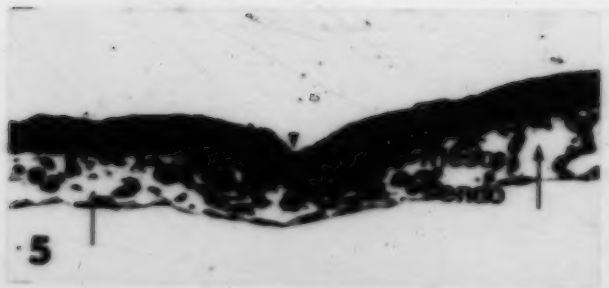
Fig. 6 Transmission electron micrograph (TEM) of thin section through Stage 5 chick embryo epiblast showing a coated vesicle (CV) with its array of bristle-like projections (arrowheads). Stained in uranyl acetate and lead citrate. X 76,800.

Fig. 7 TEM of thin section through Stage 5 chick embryo epiblast illustrating CV's in close proximity to Golgi bodies (G). Note the presence of a coated pit (arrow) on the lateral surface of the cell. Stained in uranyl acetate and lead citrate. X 37,100.

Fig. 8 TEM of thin section through Stage 5 chick embryo epiblast cell. Note the presence of immediately adjacent coated pits on the lateral surfaces of two neighboring cells (arrows). Stained in uranyl acetate and lead citrate. X 42,900.

Fig. 9 TEM of sphere of "granule fraction" yolk (y) overlying CP (arrow) on the dorsal surface of the epiblast of Stage 5 chick embryo. Stained in uranyl acetate and lead citrate. X 42,900.

Fig. 10 TEM showing coated regions of membrane (arrows) in close association Golgi associated membranes (G) in Stage 5 chick embryo epiblast. Stained in uranyl acetate and lead citrate. X 74,250.



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(Fig. 9). CV's were also frequently observed in close proximity to Golgi associated membranes (Fig 10 and Fig. 7).

Transmission electron microscopy (TEM) of cultured explants from epiblast, mesoderm and endoblast of the Stage 5 chick embryo, revealed the presence of CP's and CV's under these conditions as well.

3) Cationized Ferritin Experiments with Stage 5 Embryos

In an effort to characterize the surface charge of cell membranes of the Stage 5 chick embryo and in particular the CP's, the positively charged ultrastructural marker cationized ferritin (CF) was employed. TEM examination of the CF solution (diluted to 0.0025 mg/ml in PBS) dried on a grid, confirmed that the solution consisted of a majority (64%) of individual or paired CF molecules (Fig. 11), and aggregates of 3 molecules or greater comprised a minority of the CF sample (36%).

When living Stage 5 embryos were immersed in 0.25 mg/ml CF for 1 minute at room temperature, then rinsed and fixed, subsequent TEM examination of thin sections showed an abundance of CF bound to the dorsal surface of the epiblast (Fig. 12) and relatively little bound to the ventral surface of the endoblast (Fig. 13). No CF was seen in the tissue space between the epiblast and endoblast, presumably due to a failure to penetrate into these areas. Controls treated with native ferritin

did not bind this label to any surface, even when the concentration was increased to five times the concentration of CF used in the experiment. Therefore, the binding properties of CF appear to be solely due to its cationic character.

The vast majority of CF bound to cell membranes appeared in clumps and although CF clumps were present within CP's, they were not preferentially bound there. CF molecules were also observed bound to extracellular globules of granule-fraction yolk (Fig. 14).

No consistent variations in the density or pattern of CF binding was detected with regard to the particular area of the dorsal epiblast surface sectioned, with the notable exception of the primitive streak. No CF was observed bound to the cell membranes in the deepest portions of the primitive streak, even though no physical barrier to penetration of the label into this area was evident, and bound CF was abundant in other areas of the same epiblast.

To investigate the possibility of a post-binding rearrangement of CF (i.e. initial diffuse binding followed by clumping of bound ligand), Stage 5 embryos were immersed in 0.25 mg/ml CF in PBS for 1 minute at 4°C, followed by a rinse and fixation in glutaraldehyde at 4°C. TEM examination of these embryos (Fig. 15) yielded essentially the same results as the experiment conducted at room temperature (i.e. the CF was clumped). A second

approach undertaken in this direction was fixation of the embryos in glutaraldehyde, followed by a five minute rinse in 5% glycine to neutralize residual free aldehydes, then exposure to 0.25 mg/ml CF for 1 minute at room temperature. Again the CF binding characteristics in this experiment (Fig. 16) were essentially the same as for living Stage 5 embryos. Therefore, in living embryos, the CF does not initially bind diffusely to the cell membranes but rather in clumps.

4. Comparative CF Labeling of Stage 1, 3 and 5 Embryos

Looking for possible developmental changes in CF binding characteristics, Stages 1, 3, and 5 chick embryos were utilized. The concentration of CF in which embryos were immersed was reduced to 0.12 mg/ml in PBS for these experiments, and in one half of the embryos in each experimental group of six, the endoblast or hypoblast was removed prior to any chemical treatments. These modifications were instituted to ascertain whether a concentration-dependent variation in CF binding characteristics was observable at these concentrations (0.25 mg/ml vs. 0.12 mg/ml), and to expose the ventral surface of the epiblast, with its developing basal lamina, and migrating mesoderm cells to CF.

No differences in CF binding characteristics were observed between the developmental stages of embryos studied. Also, the reduced CF concentration did not

Fig. 11 TEM illustrating CF solution diluted to 2.5 X 10^{-4} mg/ml and dried on a formvar coated grid. Note the majority of individual particles. X 111,300.

Fig. 12 TEM depicting the dorsal surface of an epiblast cell in a Stage 5 chick embryo treated with 0.25 mg/ml CF for 1 minute at room temperature. Note the abundance of clumped CF particles. X 29,700.

Fig. 13 TEM showing the ventral surface of an endoblast cell in a Stage 5 chick embryo treated with 0.25 mg/ml CF for 1 minute at room temperature. Note the few CF particles present within the CP (arrow). X 55,700.

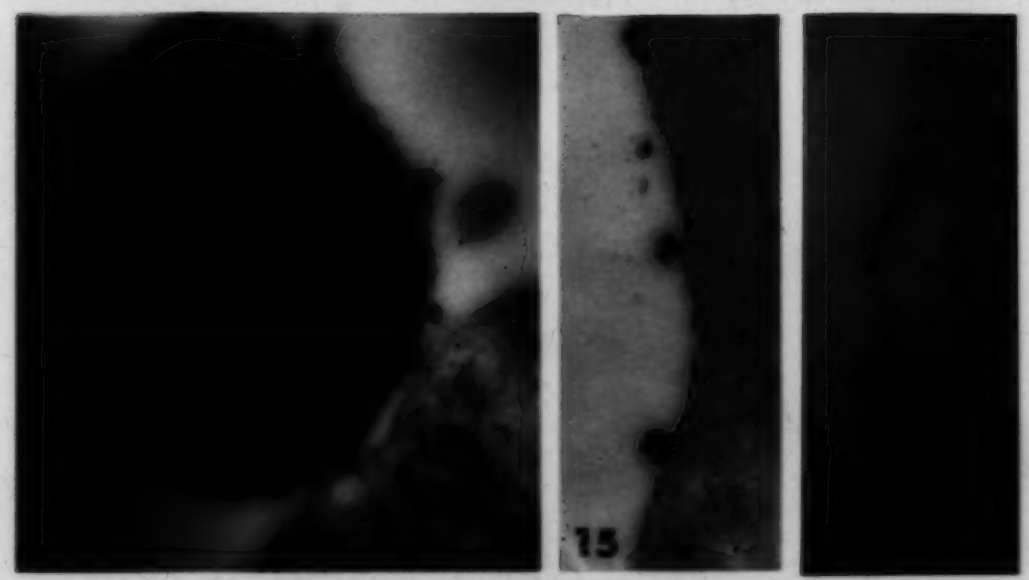
Fig. 14 TEM illustrating a sphere of "granule fraction" yolk (Y) overlying the dorsal surface of an epiblast cell in a Stage 5 chick embryo treated with 0.25 mg/ml CF for 1 minute at room temperature. Note the CF particles bound to the periphery of the yolk sphere. Stained with uranyl acetate and lead citrate. X 55,700.

Fig. 15 TEM depicting the dorsal surface of an epiblast cell in a Stage 5 chick embryo treated with 0.25 mg/ml CF for 1 minute at 4°C. Note the clumped distribution of CF particles. X 55,700.

Fig. 16 TEM of the dorsal surface of an epiblast cell from a Stage 5 chick embryo, which was fixed in glutaraldehyde prior to exposure to 0.25 mg/ml CF for 1 minute at room temperature. X 55,700.

Fig. 17 TEM of the dorsal surface of an epiblast cell from a Stage 5 chick embryo treated with 0.12 mg/ml CF for 10 minutes at 37°C following glutaraldehyde fixation. X 55,700.

Fig. 18 TEM showing the dorsal surface of an epiblast cell from a Stage 3 chick embryo treated with 0.12 mg/ml CF for 10 minutes at 37°C prior to glutaraldehyde fixation. Note the clumped distribution of CF particles bound to the plasma membrane and the "granule fraction" yolk spheres (Y), as well as the membrane indentations suggestive of developing CP's (arrows). X 55,700.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appear to affect the binding density or distribution of CF on Stage 5 embryos (Fig. 17). The majority of CF bound to cells fixed prior to CF exposure, after a 10 minute incubation in CF at 37°C, was patched (i.e. clumped) on the dorsal surface of the epiblast, sometimes in CP's and other invaginations. The very small amount which was bound to other cell surfaces was negligible in comparison, but when seen was also patched.

Embryos exposed to CF prior to fixation were treated in one of two ways. One group was immersed in CF for 10 minutes at 37°C, then fixed in glutaraldehyde. TEM examination of these embryos revealed CF bound predominantly in patches, almost exclusively to the dorsal surface of the epiblast and overlying yolk granules (Fig. 18). That CF was being endocytosed in CV's was also apparent after this incubation, as CF was observed in CP's, CV's and uncoated irregularly-shaped, membrane-bound vesicles (Figs. 19 and 20). Also, CF was frequently observed to be sandwiched between cells of the epiblast (Figs. 21 and 22).

A second group of embryos was immersed in CF for 10 minutes at 37°C, then rinsed with PBS at 37°C for a further 30 minutes before fixation. This experiment was designed to detect any rapid clearance of CF from the cell surface, and to trace the fate of endocytosed CF on a longer term basis than previously attempted. Again the majority of CF bound to the embryos was observed

on the dorsal surface of the epiblast and between epiblast cells. Therefore, CF is not rapidly cleared from the cell surface. CF was also observed in CV's, uncoated, irregularly-shaped membrane-bound vesicles and multivesicular bodies (MVB's), as shown in Fig. 23. Some of the membrane enclosed areas in which CF was seen also contained yolk granules (Fig. 24).

5) CF-Treated Tissue Cultures

Epiblast, mesoderm and endoblast tissue culture explants were flooded with 0.25 mg/ml CF at 4°C for 10 minutes prior to glutaraldehyde fixation. CF molecules were clustered on the cell surfaces of all three types of tissue culture with bare expanses of membrane in between (Figs. 25 and 26), except when seen between two cells where stretches of monolayer or bilayers of CF particles were commonly observed (Figs. 27 and 28). No consistent differences between tissue types, with regard to CF binding, were noted. CF was observed within CP's but not noticeably concentrated there.

When all three tissue types in culture were exposed for 10 minutes to 0.25 mg/ml CF at 37°C, CF particles were present in clumps on the medium-facing plasma membranes and in spaces between cells in all cases. CF was internalized by all three tissue types via endocytosis involving CP's and CV's. CF was also observed in uncoated, irregularly-shaped vesicular structures near the cell

Fig. 19 TEM depicting the dorsal surface of an epiblast cell in a Stage 5 chick embryo treated with 0.12 mg/ml CF for 10 minutes at 37°C prior to glutaraldehyde fixation. Note the clumps of CF particles in and overlying the CP (arrow). X 55,700.

Fig. 20 TEM illustrating the presence of CF in an uncoated, irregularly-shaped vesicle in an epiblast cell of a Stage 1 chick embryo treated with 0.12 mg/ml CF for 10 minutes at 37°C prior to glutaraldehyde fixation. X 55,700.

Fig. 21 TEM showing CF particles sandwiched between the lateral surfaces of two epiblast cells in a Stage 5 chick embryo treated with 0.12 mg/ml CF for 10 minutes at 37°C prior to glutaraldehyde fixation. X 55,700.

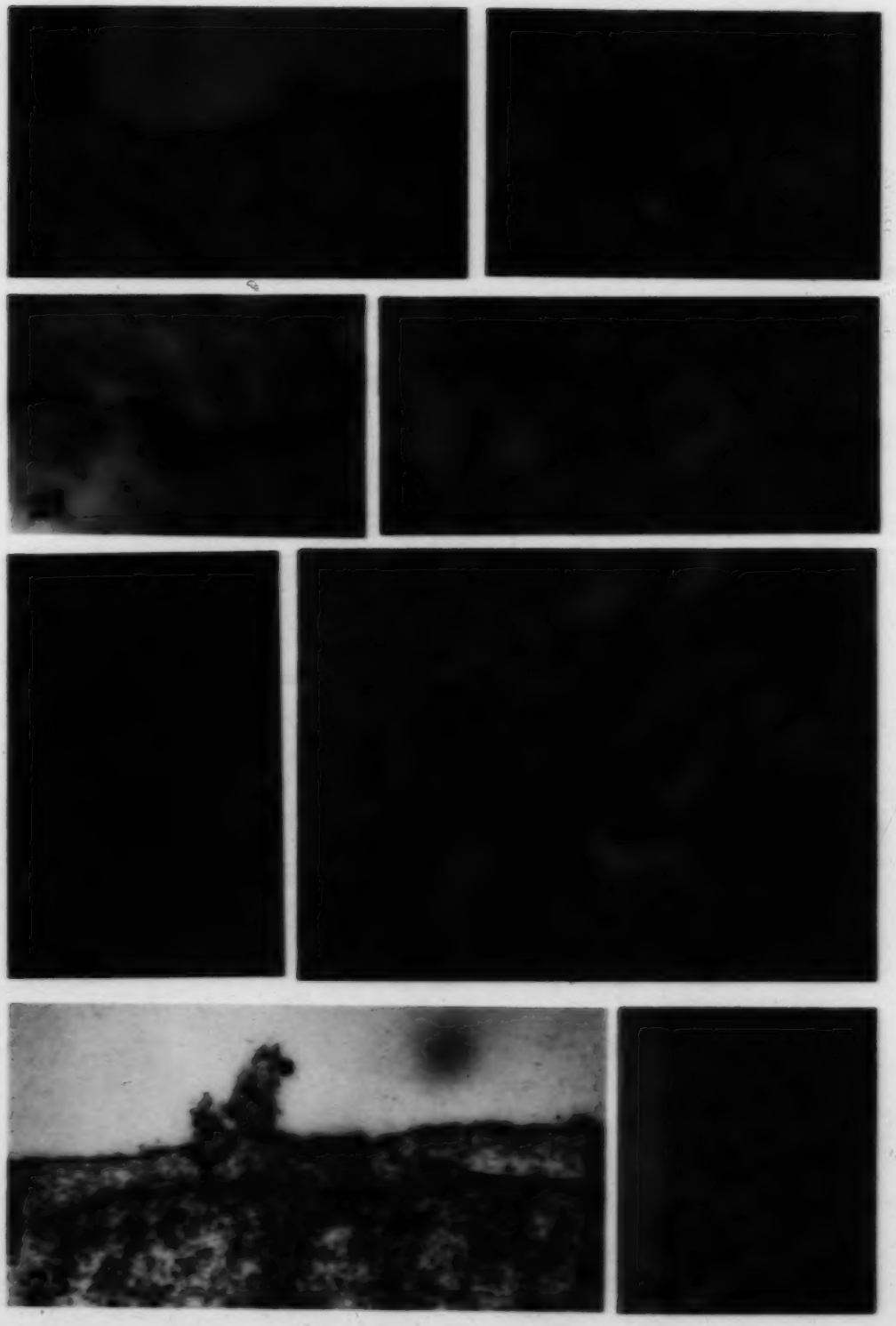
Fig. 22 TEM showing CF particles sandwiched between the lateral surfaces of two epiblast cells in a Stage 5 chick embryo, treated with 0.12 mg/ml CF for 10 minutes at 37°C prior to glutaraldehyde fixation. X55,700.

Fig. 23 TEM depicting CF particles present within a multivesicular body (MVB) in an epiblast cell of a Stage 3 chick embryo, treated with 0.12 mg/ml CF for 10 minutes at 37°C, followed by incubation in CF-free PBS for 30 minutes at 37°C and glutaraldehyde fixation. X 55,700.

Fig. 24 TEM of an uncoated, irregularly-shaped membrane-enclosed area, containing both CF particles and granule fraction yolk (Y), in an epiblast cell of a Stage 5 chick embryo. The embryo was treated with 0.12 mg/ml CF for 10 minutes at 37°C, followed by incubation in CF-free PBS for 30 minutes at 37°C and glutaraldehyde fixation. X 55,700.

Fig. 25 TEM illustrating a mesoderm tissue culture treated with 0.25 mg/ml CF at 4°C for 10 minutes prior to glutaraldehyde fixation. Note the clumps of CF bound to the plasma membrane both within and outside the CP (arrow). Post-stained with uranyl acetate and lead citrate. X 55,700.

Fig. 26 TEM illustrating an epiblast tissue culture treated with 0.25 mg/ml CF at 4°C for 10 minutes prior to glutaraldehyde fixation. Note the discrete clumps of membrane-bound CF (arrows) X 55,700



surfaces (Fig. 29). After one hour of CF exposure at 37°C, CF was still found in these same types of locations, as well as further from the cell surface in large MVB's (Fig. 30).

Serum free medium (SFM) was substituted for the normal medium in 24 hours old tissue cultures, followed by further 24 hour incubation. Culture dishes were then flooded with 0.25 mg/ml CF for 10 minutes at 37°C. This experiment was carried out to determine whether serum components were binding to the surfaces of cells in culture and imparting a negative charge to them, thereby influencing the above results. The initial 24 hours incubation in serum-containing medium (SCM) facilitated attachment and spreading of the tissue explants on the substratum prior to incubation in SFM.

Very little difference in the overall morphology of the cultures was observed when comparing individual cultures before and after SFM. The only noticeable difference was that after SFM more small gaps were evident in the sheets of spread cells, i.e. more internal cells were not contacting neighboring cells on all sides in the plane of the sheet. No difference with regard to CF binding and endocytosis, following a 10 minute incubation in CF at 37°C, were evident between the three tissue types, or cultures grown in either SFM or SCM were noted (Fig. 31).

Fig. 27 TEM of endoblast tissue culture treated with 0.25 mg/ml CF at 4°C for 10 minutes prior to glutaraldehyde fixation. Note the patchy distribution of CF particles on the medium facing membrane surface (arrow) and the more uniform monolayer of CF between cells (arrowhead). Stained with uranyl acetate. X 55,700.

Fig. 28 TEM of mesoderm tissue culture treated with 0.25 mg/ml CF at 4°C for 10 minutes prior to glutaraldehyde fixation. Note the patchy distribution of CF particles on the medium-facing membrane surface (arrow) and the interrupted bilayer of CF between cells (arrowhead). Stained with uranyl acetate. X 55,700.

Fig. 29 TEM of endoblast tissue culture treated with 0.25 mg/ml CF for 10 minutes at 37°C prior to glutaraldehyde fixation. Note CF in uncoated structure near cell surface (arrow). X 55,700.

Fig. 30 TEM of epiblast tissue culture treated with 0.25 mg/ml CF for 1 hour at 37°C. CF is present in MVB and is apparently transported there via endocytic vesicles fusing with the MVB (arrow). Stained with uranyl acetate. X 55,700.

Fig. 31 TEM of serum-free endoblast tissue culture treated with 0.25 mg/ml CF for 10 minutes at 37°C. Note the presence of CF particles clumped on the cell surface, within a CV (arrow) and within uncoated irregularly-shaped structure (arrowhead). X 55,700.

Fig. 32 TEM of cell on the dorsal surface of the epiblast of a Stage 5 chick embryo, after treatment with cytochalasin B and 0.25 mg/ml CF for 5 minutes at 37°C followed by glutaraldehyde fixation. Note the CF clumps in CP's (arrows). X 55,700.

6) Microfilaments and Endocytosis of CP

In an effort to determine whether microfilaments are involved in the endocytosis of CP in the Stage 5 embryo, specimens were initially immersed in 2 x 10^{-5}M cytochalasin B in 0.1% v/v DMSO. This treatment was carried out for 2 hours prior to CP exposure. CP was then added to all solutions at a final concentration of 0.25 mg/ml, and the embryos were incubated. After these incubations at 37°C, all embryos were thoroughly rinsed and fixed.

No differences were apparent between control and cytochalasin B treated embryos of the same incubation time. In both groups, by 5 minutes incubation time in the presence of CP, CP was bound in clumps, mainly on the dorsal surface of the epiblast. Also, CP was often seen between closely apposed surfaces and CV's and CP's near the cell surface (Fig. 32 and Fig. 33). At 15 minutes incubation the cell surface binding characteristics remained the same, CP was present in CV's and uncoated, irregularly-shaped vesicular structures (Fig. 34 and Fig. 35). By 30 minutes incubation time, CP was in all the above mentioned structures, as well as in MVB's (Fig. 36) and in CV's deep within the cytoplasm, occasionally in close proximity to Golgi elements (Fig. 37). Larger amounts of CP had accumulated inside membrane-bound compartments at 30 minutes than at 15 minutes (Fig. 38).

Fig. 33 TEM depicting the dorsal surface of the epiblast in a Stage 5 chick embryo after treatment with cytochalasin B and 0.25 mg/ml CP for 5 minutes at 37°C, followed by glutaraldehyde fixation. Note CP apparently endocytosed and present within an uncoated vesicle (arrow). X 55,700.

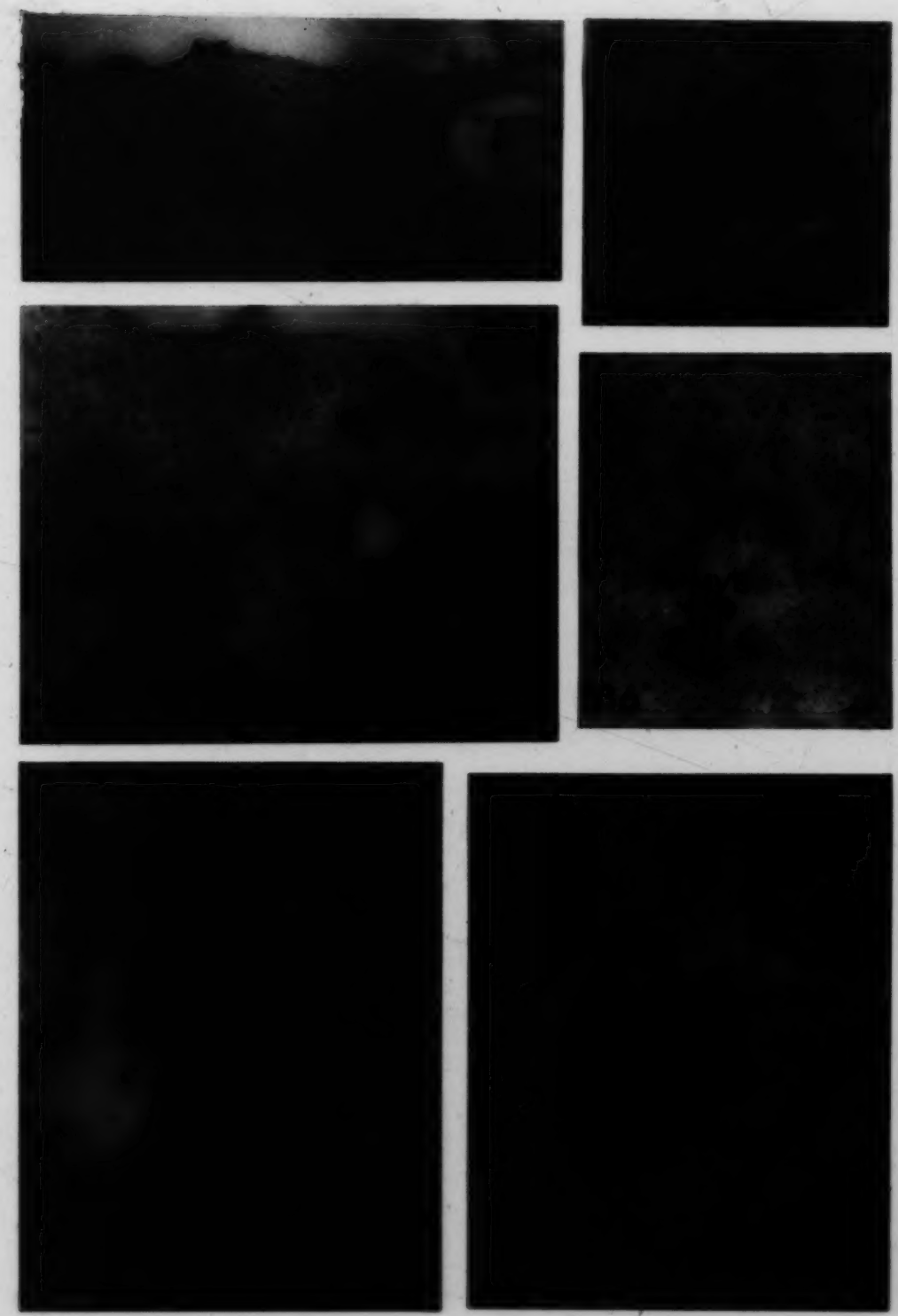
Fig. 34 TEM illustrating the dorsal surface of the epiblast in a Stage 5 chick embryo after treatment with cytochalasin B and 0.25 mg/ml for 15 minutes at 37°C, followed by glutaraldehyde fixation. Note CP apparently undergoing endocytosis in CP's (arrows) and CP present deeper in the cytoplasm within uncoated, irregularly-shaped vesicular structures (arrowhead). X 55,700.

Fig. 35 TEM showing a CP-containing, irregularly-shaped vesicular structure (arrow) within an epiblast cell of a Stage 5 chick embryo after treatment with cytochalasin B and 0.25 mg/ml CP for 15 minutes at 37°C, followed by glutaraldehyde fixation. X 55,700.

Fig. 36 TEM of area within epiblast cell in a Stage 5 chick embryo after treatment with cytochalasin B and 0.25 mg/ml CP for 30 minutes at 37°C, followed by glutaraldehyde fixation.

Fig. 37 TEM depicting area within epiblast cell in a Stage 5 chick embryo after treatment with cytochalasin B and 0.25 mg/ml CP for 30 minutes at 37°C, followed by glutaraldehyde fixation. Note the CP particles present within uncoated, irregularly-shaped structures (arrowheads) and a CV (arrow) very close to a Golgi complex (G). X 55,700.

Fig. 38 TEM illustrating area within an epiblast cell in a Stage 5 chick embryo after treatment with cytochalasin B and 0.25 mg/ml CP for 30 minutes followed by glutaraldehyde fixation. Note the massive accumulation of CP particles within a membrane-bound compartment (arrow) and adjacent structure (arrowhead). X 55,700.



7) Microtubules and Endocytosis of CF

To ascertain whether or not microtubules (MT's) are involved in the endocytosis of CF in the Stage 5 chick embryo, the MT disrupting agents colchicine and vinblastine were employed. Embryos in the experimental groups were immersed in either 2 x 10^{-5}M vinblastine or colchicine at room temperature for 2 hours immediately prior to CF exposure. Control embryos were immersed in PBS for the same length of time. CF was then added to each embryo-containing solution to a final concentration of 0.25 mg/ml, and the embryos were incubated at 37°C for 5, 15 and 30 minutes respectively prior to fixation.

No differences with regard to CF binding and endocytosis were observed between experimental and control embryos of identical incubation times. In all cases after 5 minutes incubation time CF was bound mainly to the dorsal surface of the epiblast in clumps both within and outside CP's, and inside CV's close to the cell surface (Fig. 39 and Fig. 40). After 15 minutes incubation in CF-containing solution, CF was observed as at 5 minutes, as well as in uncoated, irregularly-shaped vesicular structures and in CV's deeper within the cell (Fig. 41). By 30 minutes exposure time CF was observed in all previously mentioned sites, as well as in MVB's (Fig. 42), and CV's deep within the cytoplasm, occasion-

ally associated with Golgi elements (Fig. 43). Some observations suggested these MVB's may have originated and/or enlarged by successive fusions of endocytic vesicles (Figs. 44, 30 and 35).

8) Horseradish Peroxidase (HRP) Experiments

HRP was considered as a possible alternative to CF as an endocytic pathway tracer in the Stage 5 chick embryo. HRP (2 mg/ml) was dissolved in PBS and embryos were immersed in this solution at 4°C for 10 minutes prior to fixation. Following DAB treatment and further TEM preparation, no reaction product was observed to be bound anywhere on the cell surfaces or within the cells of any embryo.

The possibility that HRP could be endocytosed without binding to the cell surface (i.e: bulk fluid-phase endocytosis) was investigated as follows. Embryos were immersed in HRP (2 mg/ml) solution at 37°C for either 10 minutes or 30 minutes prior to fixation. After DAB exposure and routine TEM preparation, again no reaction product was observed on the cell surfaces or intracellularly.

9) Phosvitin (PV) Experiments

The presence of PV- containing yolk granules immediately apposed to the cell membranes of the Stage 5 chick embryo appeared to bear a relationship to the

Fig. 39 TEM of the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with vinblastine and 0.25 mg/ml CF for 5 minutes at 37°C, followed by glutaraldehyde fixation. Note the clumped distribution of CF on the cell surface, and presence of CF particles in uncoated, irregularly-shaped compartments (arrowhead) and in CV (arrow). X 55,700.

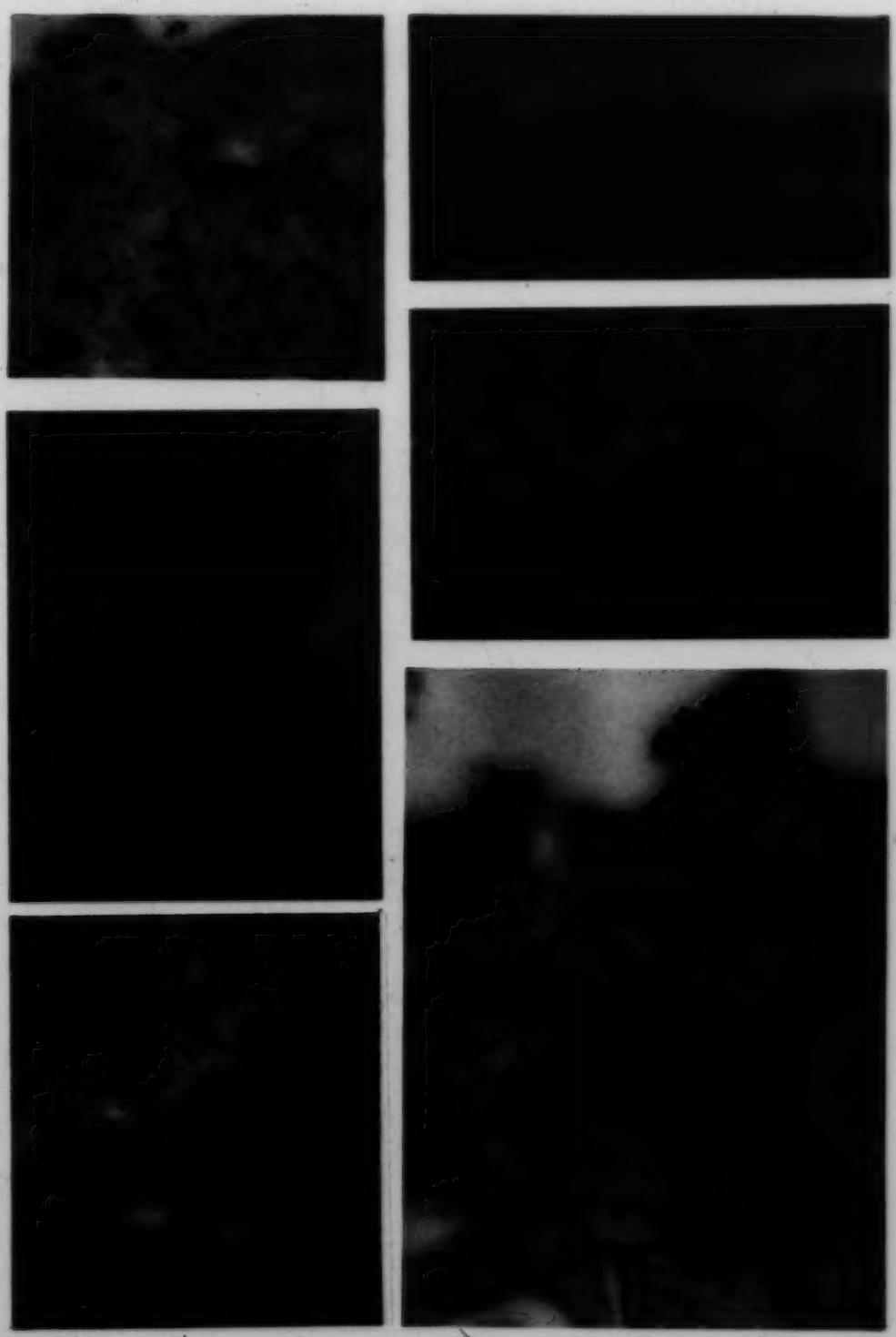
Fig. 40 TEM showing the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with colchicine and 0.25 mg/ml CF for 5 minutes at 37°C, followed by glutaraldehyde fixation. Note the clumped distribution of CF particles bound to the cell surface and the presence of CF within an irregularly-shaped compartment (arrow). X 55,700.

Fig. 41 TEM depicting the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with colchicine and 0.25 mg/ml CF for 15 minutes at 37°C, followed by glutaraldehyde fixation. Note the abundance of CF particles in uncoated, irregularly-shaped structures within the cytoplasm (arrows). X 55,700.

Fig. 42 TEM illustrating area within epiblast cell in a Stage 5 chick embryo after treatment with colchicine and 0.25 mg/ml CF for 30 minutes at 37°C, followed by glutaraldehyde fixation. Note the presence of numerous CF particles within MVB. X 55,700.

Fig. 43 TEM of area within epiblast cell in a Stage 5 chick embryo after treatment with colchicine and 0.25 mg/ml CF for 30 minutes at 37°C, followed by glutaraldehyde fixation. Note the presence of CF particles in uncoated, irregularly-shaped structures (arrowheads) and CV (arrow). X 55,700.

Fig. 44 TEM showing the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with colchicine and 0.25 mg/ml CF for 30 minutes at 37°C, followed by glutaraldehyde fixation. Note the presence of CF particles in the MVB and in an endocytic vesicle (arrow) apparently undergoing fusion with the MVB. X 55,700.



location of CP's and CV's in these cells. Therefore, the possibility that a general increase in extracellular PV concentration could cause a concomitant increase in the frequency of occurrence of CP's and CV's in exposed cells was investigated.

Both embryos and 24 hour old cultures of each tissue type were immersed in either 0.05 mg/ml PV or 0.5 mg/ml PV for 15 minutes at 37°C. Control cells were incubated in PBS for 15 minutes at 37°C. Following these treatments fixation and further TEM preparation were carried out as usual. An increased abundance of CP's or CV's was not observed in conjunction with increased extracellular PV concentration in either embryos or tissue culture.

10) PV-Ferritin Conjugate Experiments

Whole stage 5 embryos and 24 hour old tissue cultures were immersed in PV-ferritin conjugate for 10 minutes at either 37°C or 4°C. In the embryos, the electron-dense ferritin label was observed bound to the dorsal surface of the epiblast (Fig. 45) and the ventral surface of the endoblast (Fig. 46) at 4°C. Epiblast, mesoblast and endoblast cells in culture (24 hours) also bound PV-ferritin to their cell surfaces at 4°C (Fig. 47 and Fig. 48). On all mentioned cell surfaces, the conjugate was bound to uncoated membrane as well as CP's. No endocytosis of the conjugate was evident at 4°C.

Fig. 45 TEM depicting the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate for 10 minutes at 4°C, followed by glutaraldehyde fixation. Note the clumps of ferritin label bound to the plasma membrane in a CP (arrow) and on microvillus (arrowhead). X 55,700.

Fig. 46 TEM of the ventral surface of an endoblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate for 10 minutes at 4°C, followed by glutaraldehyde fixation. Note the four particles of ferritin label inside the CP (arrows). X 55,700.

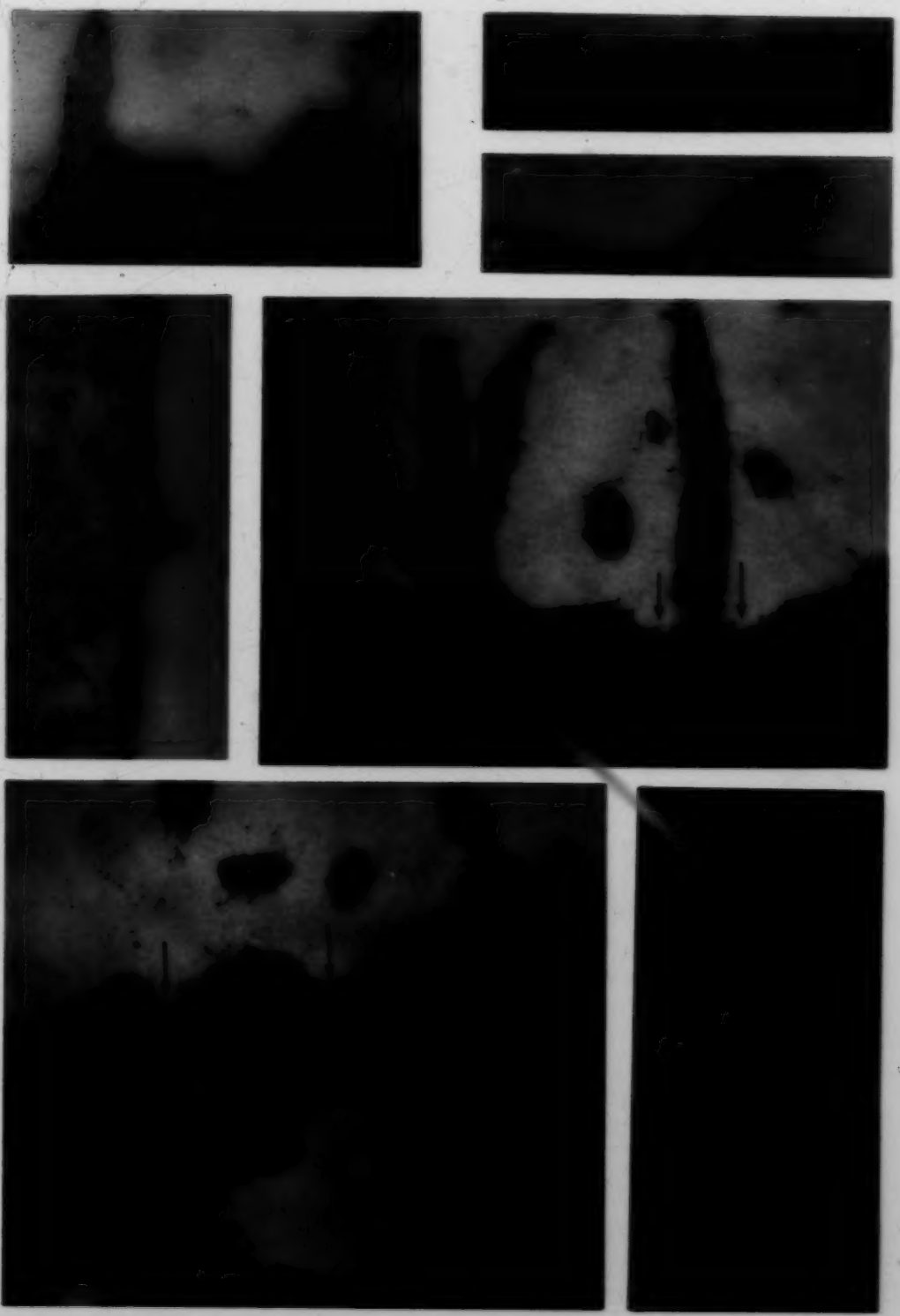
Fig. 47 TEM illustrating epiblast tissue culture treated with PV-ferritin conjugate for 10 minutes at 4°C, followed by glutaraldehyde fixation. Note the clumps of ferritin label (arrows) on the medium-facing surface of the cell. X 55,700.

Fig. 48 TEM showing mesoderm tissue culture treated with PV-ferritin conjugate for 10 minutes at 4°C, followed by glutaraldehyde fixation. Note the clump of ferritin label (arrow) on the medium-facing surface of the cell. X 55, 700.

Fig. 49 TEM of the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate for 10 minutes at 37°C, followed by glutaraldehyde fixation. Note the presence of ferritin label on the cell surface exclusively overlying CP's (arrows). Stained with uranyl acetate. X 55,700.

Fig. 50 TEM of the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate for 10 minutes at 37°C, followed by glutaraldehyde fixation. Note the presence of ferritin label on the cell surface exclusively overlying CP's (arrows), and within the cell contained in an irregularly-shaped structure along with what appears to be "granule-fraction" yolk (arrowheads). Stained with uranyl acetate. X 55,700.

Fig. 51 TEM depicting the ventral surface of an endoblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate for 10 minutes at 30 minutes at 37°C, followed by glutaraldehyde fixation. Note ferritin label in CP's (arrow). X 55,700.



At 37°C, conjugate undergoing endocytosis was observed in CP's, CV's (Figs. 49 thru 53) and inside uncoated, irregularly-shaped vesicular structures, some of which were MVB's which also contained yolk granules (Fig. 54 and Fig. 55). On the cell surface, PV-ferritin was bound almost exclusively in CP's, but occasionally to extracellular yolk granules as well (Fig. 56). Controls exposed to native ferritin at similar concentrations (0.25-5 mg/ml) reveal no binding or endocytosis of the label.

11) Specificity of PV-Ferritin Conjugate Binding

An attempt to determine the specificity of the interaction between cell surface binding sites and PV portion of PV-Ferritin conjugate was made. In the presence of not less than a thirty fold excess of "free" PV, the conjugate did not bind to any surface of the Stage 5 chick embryo, nor was it endocytosed during a 10 minute incubation at 37°C. However, when the same experiment was performed with not less than a thirty fold excess of bovine serum albumin (type V), the conjugate was visualized by TEM to be bound to the cell surfaces of the dorsal epiblast and ventral endoblast. In these embryos the conjugate was observed on the cell surface almost exclusively in CP's (Fig. 57), and around the base of microvilli, where CP's often form (see Figs.

Fig. 52 TEM illustrating epiblast tissue culture treated with PV-ferritin conjugate for 10 minutes at 37°C, followed by glutaraldehyde fixation. Note the presence of conjugate label inside CV (arrow). X 55,700.

Fig. 53 TEM of the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate for 10 minutes at 37°C, followed by glutaraldehyde fixation. Conjugate label is present in CV (arrow) located within cellular extension. X 55,700.

Fig. 54 TEM showing the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate for 10 minutes at 37°C, followed by glutaraldehyde fixation. Note the presence of the conjugate label in vesicular structure closely associated with "granule fraction" yolk (arrows) and in irregularly-shaped vesicular structures (arrowheads). X 55,700.

Fig. 55 TEM depicting an area of an endoblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate for 10 minutes at 37°C, followed by glutaraldehyde fixation. Note the presence of the conjugate label closely associated with "granule fraction" yolk (arrows) inside vesicular structure. X 55,700

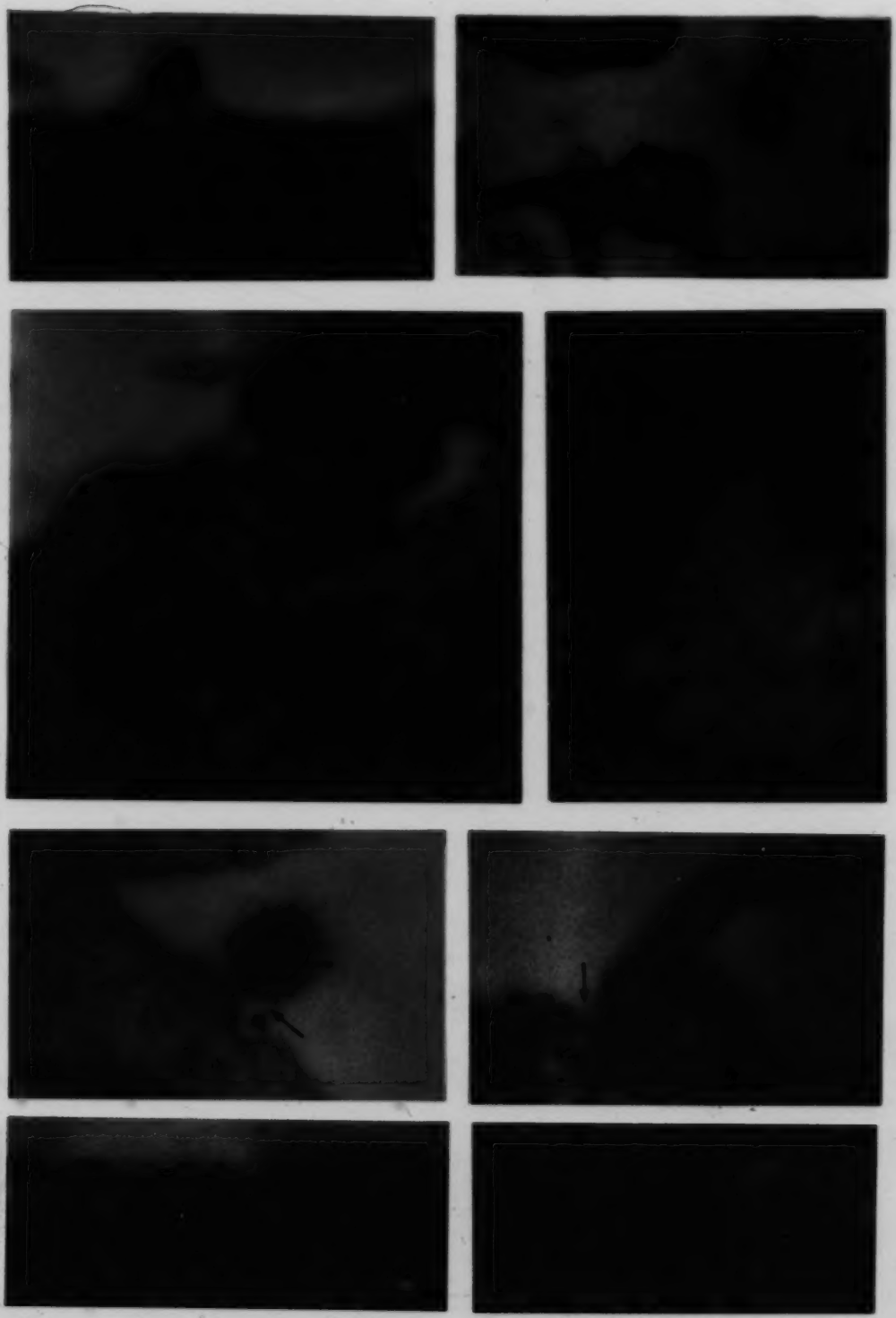
Fig. 56 TEM of the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate for 10 minutes at 37°C, followed by glutaraldehyde fixation. Note the ferritin particles visible only in the immediate area of the "granule fraction" yolk (arrow). X 55,700.

Fig. 57 TEM of the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate in the presence of a 30X excess of bovine serum albumin (BSA) for 10 minutes at 37°C, followed by glutaraldehyde fixation. Note the ferritin particles bound to a CP (arrow) and around the base of the microvillus (mv), shown by arrowhead. X 55,700.

Fig. 58 TEM of the ventral surface of an endoblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate in the presence of a 30X excess of BSA for 10 minutes at 37°C, followed

by glutaraldehyde fixation. Note the ferritin particles visible only in the CV (arrow). X 55,700.

Fig. 59 TEM illustrating the dorsal surface of an epiblast cell in a Stage 5 chick embryo after treatment with PV-ferritin conjugate in the presence of a 30-fold excess of BSA for 10 minutes at 37°C, followed by glutaraldehyde fixation. Note the ferritin label present inside uncoated vesicular structure (arrow). X 55,700.



DISCUSSION

The cell surface is known to be a critical determinant of the nature and occurrence of phenomena such as cell-to-cell and cell-to-substrate adhesion, cell locomotion and intercellular recognition. For these reasons, attempts have been made to characterize and correlate various properties of the cell surface and observable cellular behavior. The early chick embryo provides a good system for such experimentation, as it is readily available, easy to process for scanning and transmission electron microscopy, and its pattern of morphogenesis is reasonably well documented.

That the cells of the early chick embryo possess a net negative charge has previously been established by cell electrophoresis (Zalik et al, 1972). This study also showed that there is a progressive reduction in negative cell surface charge from Stage 1 through Stage 5 embryos, and that only a very minor, if any, contribution is made by sialic acid to the negative cell surface charge. Furthermore, exposure to high concentrations of Ca^{++} ions has been shown to be capable of effecting a charge reversal in these cells (Harris and Zalik, 1974).

In the present study, it has been found that changes in the cell surface charge, as detected by

cationized ferritin (CF) binding, appear to be related to morphogenetic events, in particular the migration of epiblast cells down through the primitive streak. The dorsal surface of the epiblast was found to be the only cell surface in the Stage 1 through Stage 5 chick embryo which bound CF to any significant degree. Also, the anionic cell surface moieties visualized by CF binding are present in a clumped or patched distribution at 37°C, except when CF was observed bound between cells in regions of close intercellular apposition where a more ordered distribution was prevalent.

It is likely that this distribution was not due to a post-binding rearrangement of the ligand-cell surface component complexes, as is true in the majority of multivalent ligand-receptor complexes studied (for review, see Nicolson et al, 1977), but rather reflects their native distribution in the plane of the membrane. This is suggested by the fact that identical results were obtained using cells with reduced membrane mobilities due to glutaraldehyde fixation and/or low temperature. Glutaraldehyde is known to stabilize the existing protein distribution at the time of fixation through cross-linking of amino groups, and low temperature (4°C) has been found to prevent the post-binding rearrangement of receptor-bound LDL and 2 macroglobulin in human fibroblasts (Anderson et al, 1977; Maxfield et

al, 1979). Although the evidence in favor of a natively patched distribution of negative charge is substantial, the functional significance of this arrangement remains obscure.

Precise quantification of CF binding was not attempted in the present study, but by visual inspection, no progressive decline or increase in negativity of the surface charge of the dorsal surface of the epiblast, from Stage 1 through Stage 5, was evident. However, the decrease in average cell surface charge in dissociated embryos of increasing developmental stages, as reported in the previously mentioned cell electrophoresis study, could be due to the increasing proportion of cells which are undergoing or have undergone the transformation from epiblast to mesoderm or endoblast. As previously stated, this transformation is accompanied by a reduction in negative cell surface charge.

When the newly formed endoblast and mesoderm were removed from the embryo, explanted and cultured individually, they regained a degree of negative cell surface charge comparable to that of the dorsal surface of the peripheral epiblast <u>in situ</u>. Apparently this was due to change in the molecular composition of the membrane in response to an altered environment. That the change was not attributable to the binding of negatively-charged serum components to the external surfaces of the membranes is indicated by the binding studies performed in serum-

free medium. The cells cultured in serum-free medium exhibited a density and distribution of CF binding indistinguishable from that of cultures which included serum.

Previous investigators have reported a number of other cell surface alterations which occur during the gastrulation process. Stage 1 through Stage 5 chick embryos show an increasing affinity for the lectin concanavalin A (con A) with age, on the dorsal surface of the epiblast. However, the ventral surface of the Stage 1 hypoblast bound con A, whereas the ventral surface of the definitive endoblast in the Stage 5 embryo did not (Hook and Sanders, 1977). The authors concluded that since the definitive endoblast was derived from the epiblast, which had a high affinity for con A, the lectin binding sites were either masked or removed from the cell surface after invagination. Although no difference in con A binding affinity was observed between invaginating and more peripheral epiblast cells, lanthanum, which does not possess a particular glycoprotein specificity, does exhibit a differential binding affinity (Sanders and Zalik, 1972). In this study, it was found that lanthanum deposited more heavily on the epiblast cells deep within the primitive groove of Stage 5 chick embryos than on the non-invaginating, more peripheral cells. Although the specificity of the lanthanum binding is in doubt, it nevertheless reflects a definite alteration in the cell

surface properties at the time of invagination, in preparation for the extensive morphogenetic movements of gastrulation. Another example of cell surface alteration during gastrulation is a change in distribution of wheat germ agglutinin (WGA) binding sites on early chick embryo cells (Sanders and Anderson, 1978). The present study has determined that an effect of a change in molecular expression on the cell surface of gastrulating chick embryo epiblast cells is to reduce the amount of negative cell surface charge as the cell invaginates, and this reduced negativity is maintained as lateral migration proceeds. This alteration occurs at about the same time as the changes described by previous authors, and may be related to the same underlying process of masking of cell surface receptors or deletion of molecules from the plasma membrane.

At this point, it is worth noting that electrical fields have been shown to exist in the gastrulating chick embryo (Jaffe and Stern, 1979), with the primitive streak area and especially Hensen's node being strongly electropositive, presumably due to the leakage of electric current through disrupted intercellular junctions in these areas. These authors suggested that the current is driven by a sodium pump in the epiblast which results in the accumulation of ions in the cavity underlying the epiblast. The results of the present study are consistent with this hypothesis in that the cell surfaces exposed to the

suggested electropositive environment did not bind CF, whereas the dorsal surface of the epiblast readily bound CF. This could be the result of the occupation of anionic sites on the cell surface through electrostatic attraction for the abundant cations in the primitive streak area and the cell-free spaces between the epiblast and endoblast. Also, further speculation along these lines is fueled by evidence that certain cells *in vitro* respond to their position in an electric field by actively migrating toward the positive pole, utilizing the normal locomotor processes of the cells (for review, see Trinkaus, 1982). This notion is further supported by experiments with early chick embryos in culture (Stern and Goodwin, 1977). Using a technique for culture of whole embryos (New, 1955), it was demonstrated that the direction of epiblast migration during gastrulation could be distorted by an applied electrical field. It is therefore possible that the electrical fields within the chick embryo play a role in the medial migration of epiblast cells, which results in invagination of cells through the primitive streak.

Endocytosis in the Early Chick Embryo

The present study has determined that following the binding of CF to living chick embryo cells, *in situ* or *in vitro*, CF is internalized in CP's and CV's. It is suggested that the CV's containing CF then fuse with various membranous structures, including MVB's and

Golgi elements, thereby transferring the enclosed ligand into these structures. In the whole embryo, this process occurred only in the epiblast cells, although CP's and CV's were observed in all tissue types of the Stage 1 through Stage 5 chick embryo. This result implies that binding of an extracellular molecule to cell surface components is a necessary prerequisite for internalization via endocytosis in these cells. This idea is further substantiated by the finding that neither native ferritin nor horseradish peroxidase are bound by the cell surface or endocytosed by any cells examined in this study. It therefore seems a reasonable deduction that bulk, fluid-phase endocytosis does not occur in early chick embryo cells of the area pellucida under these conditions. The same principle appears to be true concerning these cells in vitro, the only apparent difference being that cultured endoblast and mesoderm cells, having regained the capacity to bind CF to their surfaces, endocytosed CF in a manner indiscernible from that of epiblast cells in situ and in vitro.

Due to the absence of fluid-phase endocytosis of HRP in these cells, the possibility that cell surface components are recycled back to the plasma membrane rather than degraded and synthesized anew was not amenable to elucidation using the double-labelling technique of Thyberg et al (1981). As the nature of the CF-binding cell surface component(s) is unknown, future experiments

along these lines should concentrate on this problem, then perhaps the appropriate synthesis inhibitors could be applied in conjunction with ligand exposure to determine whether or not these components are recycled. For example, if tryptic digestion abolished CP binding to the cell surface, and binding and endocytosis of CP continued for a prolonged period of time in the presence of cycloheximide, it would be reasonable to assume that the proteinaceous CP-binding moieties were being either recycled or replenished via an intracellular storage pool. However, at present this is an area of mere speculation.

The question of whether patches of receptor-ligand complexes position themselves over existing regions of clathrin-coated membrane, or sequester clathrin coats to underly their position has been investigated in human lymphoblastiod cells (Salisbury et al, 1980). These authors concluded that clathrin was recruited to the clusters of anti-IgM-cell surface IgM complexes, because the amount of plasma membrane that was coated increased three-fold in response to antibody challenge and the increase in CP's could be accounted for by receptor-ligand clusters which had acquired coats. This conclusion may be applicable to the mechanism of endocytosis via CP's and CV's in the chick embryo, as suggested by the results of the present study. Although the proportion of plasma membrane which was clathrin-coated

did not appear to increase dramatically with exposure to and binding of ligands, it was observed with a relatively high frequency that CP's formed directly opposite one another, in adjacent areas on the lateral surfaces of neighboring cells. This observation suggests that the location of clathrin coats on the membrane is determined, at least in some cases, by an extracellular signal of some sort. Furthermore, CP's were often observed on the dorsal surface of the epiblast, in a position directly underlying spheres of "granule fraction" yolk. It is likely that these yolk spheres are bound to cell surface components, possibly including receptors in CP's, as they persisted through the various preparative washes.

"Granule fraction" yolk consists of a crystalline array of lipovitellins and phosvitins (Bellairs and Backhouse, 1972), which are proteolytic cleavage products of the maternal serum protein vitellogenin (Bergink and Wallace, 1974). During oogenesis, vitellogenin is sequestered by the developing oocyte via receptor-mediated endocytosis in CP's and CV's, and the binding of vitellogenin to its receptor on the oocyte plasma membrane is specifically mediated by its phosvitin moiety (Woods and Roth, 1979). Thus, there is a distinct possibility that phosvitin is recognized by an embryonic cell surface receptor and receptor-mediated endocytosis is involved in yolk utilization.

Although exposure of the embryonic cells to high

extracellular concentrations of phosvitin did not appear to alter the frequency of occurrence of CP's and CV's on the exposed plasma membranes, this does not rule out the possibility that localized concentrations of phosvitin, near the cell surface, could have an influence on the distribution of CP's and CV's. Other lines of evidence detailed in this study establish a definite relationship between extracellular phosvitin and receptor-mediated endocytosis via CP's and CV's. More specifically, following incubation at 37°C, the PV-ferritin conjugate was observed bound to the cell surface, almost exclusively to areas of membrane possessing clathrin coats on their cytoplasmic surfaces, and was subsequently endocytosed in CV's. The finding that at 4°C the conjugate was more frequently bound to uncoated membrane than at 37°C implies that some degree of post-binding rearrangement occurred at 37°C, resulting in the localization of the conjugate over coated membrane areas. Alternatively, or perhaps in addition to the post-binding rearrangement, the binding of conjugate to the cell surface induced the formation of a CP and subsequent CV from the underlying membrane, and this process did not readily occur at 4°C.

The eventual fate of the endocytosed PV remains unknown, except for the observed incorporation into irregularly-shaped vesicular structures, including MVB's. Previous investigators have shown that PV is the

major storage molecule for the iron necessary for development of the fertilized oocyte (Osaki et al, 1975; Hegenaur et al, 1979; Greengard et al, 1964) and may be involved in oxidative phosphorylation (Grant and Taborsky, 1966; Yoshimura et al, 1980) as a substrate of the coupling process involved in conversion of ADP to ATP. This ascribed role for phosvitin would seem to require the transfer of PV from the plasma membrane to the mitochondrial membrane without undergoing lysosomal degradation. This task could theoretically be accomplished by properties of the CV which recognize the target organelle and selectively allow fusion of the vesicle with it. However, considering the observation of PV-ferritin conjugate inside MVB's in the present study, and the fact that many MVB's are lysosomal in nature (Pasteels, 1973), it is also possible that PV is simply degraded to provide iron, phosphorous and amino acids needed for development.

A role for microfilaments and/or microtubules in cell surface receptor distribution and translocation of CV's to destinations within the cytoplasm has been suggested (Kolset et al, 1979; Salisbury et al, 1980; Mesland et al, 1981). This suggestion has arisen from both morphological observation of cytoskeletal elements in close proximity to invaginating CP's, and from studies employing chemical inhibitors of cytoskeletal function. For example, the microtubule-disrupting agent, colchicine,

inhibited the uptake and degradation of asialo-glycoproteins by rat hepatocytes by interfering with the binding of ligand to the cell surface and intracellular transport of CV's to lysosomal compartments (Kolset et al, 1979). In the same study, it was revealed that cytochalasin B, which interferes with microfilament function, inhibited degradation of ligand by decreasing the efficiency of CV transport to the lysosomes.

In the present study, no evidence was obtained to indicate that microtubules or microfilaments were involved in the distribution of CF binding sites on the cell surface, or the translocation of CV's through the cytoplasm and delivery of contents to a larger vesicular compartment. In cells treated with colchicine or cytochalasin B, endocytosis of CF in CV's did not appear different in any way from the process as it occurred in control embryos. Previous investigators have obtained similar results concerning pinocytosis in macrophages treated with cytochalasin B (Wills et al, 1972; Silverstein et al, 1977; Thyberg, 1980). Also, pinocytosis of polyvinyl pyrrolidone by rat peritoneal macrophages has been shown to be unaffected by the presence of colchicine (Pratten and Lloyd, 1979).

In general, there is conflicting evidence concerning a possible role for cytoskeletal elements in the translocation of CV's through the cytoplasm. Due to the variation of results obtained from different systems, it

is not possible at this time to make a generalization concerning the role, or absence of such, of microfilaments and microtubules in pinocytosis via CP's and CV's. It is apparent from the present study, however, that this process is not arrested by cytoskeletal inhibitors in the early chick embryo. Perhaps studies designed to measure the rate of pinocytosis of an externally applied ligand in the presence and absence of cytoskeletal inhibitors could provide useful information on the mechanism as it operates in this system. Rates of vesicle translocation through the cytoplasm may also be useful, in that they could be compared to the rate expected by simple diffusion of the CV in the cytoplasm (for review, see Ockleford and Munn, 1980).

In conclusion, it would be of interest to perform further experiments, possibly utilizing enzymes specific for cleaving certain cell surface molecules, to determine the chemical nature of the anionic moieties observed by CF binding in this study. Also, further experiments utilizing longer incubation times and lysosomal enzyme localization would be useful in determining whether the MVB's shown to contain conjugated phosvitin perform a digestive function on the internalized yolk protein, and the long term fate of phosvitin within the cell in relation to its suggested roles as an iron carrier protein (Osaki et al, 1975; Hegenaur et al, 1979;

Greengard et al, 1964), and as a substrate for oxidative phosphorylation (Grant and Taborsky, 1966; Yoshimura et al, 1980).

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